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13. ABSTRACT (Maximum 200 Words)

Using Schwann cell lines derived from neurofibromatosis (NF) patients, primary human Schwann cells, and a Schwann cell line derived from a non-NF patient, we report metabolic changes leading to chronic proliferation of NF-derived Schwann cells. The growth factor receptors c-Kit and PDGF are overexpressed while the erb B3 receptor is absent in the NF-derived cell lines. Excess arachidonate metabolism results in the secretion of thromboxane and prostaglandins; as a consequence the basal levels of intracellular cAMP are at least 10-fold elevated in the NF-derived Schwann cells. Taken as a whole, this data leads to the following metabolic scheme which is consistent with hyperproliferation and tumor formation in NF-1 patients. MAP kinase is activated via the overexpressed growth factor receptors as well as RAS pathway which is activated as a consequence of the absent neurofibromin. The hyperstimulated MAP kinase phosphorylates and activates phospholipase A2 which releases arachidonate metabolites causing the chronic elevation of cAMP which in turn sustains the overproduction of growth factor receptors. In this manner, a self-sustaining, hyperproliferative state is attained which is characteristic of NF-1. Further experiments are in progress to confirm these molecular mechanisms of Schwann cell proliferation in NF-1.

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N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

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Table of Contents

Papers Published:

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9. Lopez, T.J., M.F. Dauzvardis, and G.H. DeVries (1996) Purification and Functional Analysis of Primary Fetal Human Schwann Cells. *Soc. Neurosci. Abst.*, 22:1725.

INTRODUCTION

A. Research Subject

The subject of this investigation is Type 1 neurofibromatosis (NF-1) which is an inherited disease which occurs at a frequency of approximately 1 in 3,000. Two types of tumors are evident in this disease: relatively benign neurofibromas, which occur as subcutaneous tumors, and neurofibrosarcomas (NFS), the more invasive and aggressive type tumors which can potentially be life-threatening. The major cell type which proliferates and forms either of these tumors is the Schwann cell. Our laboratory has obtained and developed a number of different Schwann cell lines derived from neurofibrosarcomas and/or neurofibromas which we are currently using to study this disease. In addition, we have obtained benign Schwann cell tumors (Schwannomas) from patients who are not affected by NF-1 which serve as controls for the neurofibromatosis-derived Schwann cells.

B. Purpose

The purpose of this investigation is to understand the molecular mechanisms which are responsible for the abnormal proliferation of Schwann cells which characterizes NF-1. In particular, we would like to understand how the absence of neurofibromin leads to changes in intercellular signaling which ultimately lead to a sustained proliferation of Schwann cells.

C. Scope of Research

Our approach to understanding the abnormal proliferation of Schwann cells is to evaluate receptor expression in NF-derived and non-NF-derived Schwann cells. The presence of additional growth factor receptors is closely related to increased proliferative potential of Schwann cells. In the presence of the appropriate ligand to activate the receptor, these additional receptors could be responsible for the abnormal proliferation which is observed in NF-derived Schwann cells. As outlined in the original proposal, we proposed to manipulate receptor expression in order to create Schwann cells which mimic cells obtained from NF-1 patients. We also extended our investigation to ask whether or not other growth factor receptors were overexpressed in these cells and what intercellular metabolic pathways could be responsible for increased growth factor expression. We are focusing on a key regulator of intracellular metabolism, namely mitogen activated protein kinase (MAPK). The activation of this enzyme, which is integrally related to cellular proliferation, may be important in understanding growth factor receptor overexpression in particular, and in general, the molecular mechanisms of Schwann cell proliferation in NF- 1.

D. Background of Previous Work.

Our original proposal was based on the observation that both stem cell factor and its receptor KIT were expressed at abnormal levels in a NF-1 derived Schwann cell line. The rational for this research was the observation that neurofibromas contain multiple cell types including Schwann cells, perineural cells, endothelial cells, fibroblasts, and most particularly, mast cells (1,2). Mast cells are also found in the endoneurial and perineurial spaces of peripheral

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nerves (3). Mast cells are also found in close proximity to Schwann cells which myelinate peripheral nerves (4). In neurofibromatosis, mast cell numbers increase dramatically in peripheral nerves (4). These observations raised the possibility that in neurofibromatosis, mast cells both respond to and secrete the hematopoetic growth factor stem cell factor (SCF) which, in an autocrine manner, can induce mast cell development, function, and migration (5-9). In addition, the SCF secreted by mast cells could stimulate Schwann cells in a paracrine manner. The receptor tyrosine kinase KIT is the target for SCF and can be synthesized in a membrane bound or secreted form (6.10-12). Stem cell factor can also affect the proliferation and development of a number of cell types (5). Both SCF and KIT are known to be expressed in the nervous system (13). These observations are consistent with the view that mast cells, present in neurofibromas, could secrete stem cell factor which would potentially react with cKit receptors present on Schwann cells and contribute to the overall proliferative potential of the NF-derived Schwann cells. This information served as the background for our investigation of a potential role of a stem cell factor in KIT complex in the Schwann cell proliferation associated with neurofibromatosis (14).

For these studies, we utilized a human Schwannoma cell line derived from a malignant neurofibrosarcoma (termed ST88-14). The cell line was characterized as being of Schwann cell origin by virtue of its immunoreactivity with S100, Laminin, CNPase, Po, and MBP. Next, we carried out RT-PCR to detect stem cell factor in both a transformed rat Schwann cell line which was developed in our laboratory (15) as well as the ST88-14 cell line. We detected an 830 nucleotide product from cells known to contain the SCF transcript; a product of identical size which was amplified from the ST88-14 cells. Therefore, we concluded that both the neurofibroma derived ST88-14 and the transformed rat Schwann cell line expressed the mRNA for stem cell factor.

We then asked whether the stem cell factor was produced in a membrane bound or soluble form (10-12). We analyzed conditioned media from the transformed rat Schwann cell line and utilized an *in vitro* assay of induction of mast cell colonies in culture. We found that the conditioned media from the rat Schwann cell line stimulated SCF-dependent mast cell colony formation from mast cell committed progenitors. It was not possible to carry out similar assay with conditioned media from the human ST88-14 cells since the *in vitro* assay only worked with rodent cells. Therefore, we utilized a cell proliferation assay and SCF dependent cells which were cultured in the presence of concentrated conditioned media obtained from the ST88-14 cell line. We found that conditioned media from the ST88-14 cell line does not contain detectable stem cell factor. Next, we analyzed both the rat Schwann cell line and the ST88-14 cell line for the presence of membrane-bound SCF using a SCF antibody and fluorescent analysis by cell sorting (FACS analysis) (14). Both cell types gave convincing evidence of the presence of membrane-bound stem cell factor, although only the rat Schwann cell line was able to release it into the media. Therefore, we concluded that the ST88-14 human Schwannoma cell line was contained in the membrane-bound SCF. One possibility that needs to be considered is that if cKit were present on the ST88-14 cell line, it would utilize all of the SCF released into the media. Therefore, our failure to find soluble SCF needs to be interpreted with this possibility in mind.

We next examined both the normal human Schwann cells and rat Schwann cells for expression of KIT by FACS. This method allowed us to specifically distinguish florescent signals from Schwann cells in the normal human Schwann cell culture which also contained fibroblasts. The experiments convincingly demonstrated that cKit was expressed only on the

malignant Schwannoma cell line and not on normal human Schwann cells, nor on the rat Schwann cell line. Another interesting finding was that axonal contact tightly regulated the expression of SCF in Schwann cells. When the Schwann cells were associated with DRG neurites, there was absolutely no expression of stem cell factor by the Schwannoma cells. In contrast, the freshly prepared Schwann cells contained abundant immunoreactive stem cell factor (14).

In summary, this background work demonstrated for the first time that stem cell factor and its receptor KIT were coexpressed on a single cell line derived from a neurofibromatosis patient. These findings have significant implications in terms of understanding the proliferative potential of Schwann cells, since increased growth factor expression is related to increased proliferation. Although we did not detect soluble stem cell factor, it is possible that all the stem cell factor produced by the human Schwannoma cell line was being utilized to activate the cKit receptor. It is important to note that activation of cKit receptor, which is a member of the general family of receptors which also includes the PDGF receptor, can have significant consequences terms of activation of intercellular transduction pathways leading to proliferation. A key intercellular transducer is RAS, which is small GTPase activated by binding the nucleotide GTP. The GTP then is hydrolyzed GDP with consequent inactivation of RAS. Part of the neurofibromin molecule known as the GTPase activating protein (GAP) domain assists in the deactivation of RAS. When NF is defective, chronic activation of RAS would be expected to lead to the hyperproliferative state of Schwann cells. In addition to the GAP domain on neurofibromin, there are other intracellular gaps which are regulated by phosphorylation. Activation of cKit, which is a member of the PDGF family of growth factor receptors, leads to phosphorylation and deactivation of intracellular GAPs. In concert with the loss of neurofibromin, phosphorylation of GAP due to the abnormal production of growth factor receptors and the activation of these receptors could exacerbate the proliferative potential of Schwann cells.

This initial work then provided a rational for looking more deeply into the question of abnormal growth factor receptor expression. Although we first utilized the rational of mast cell produced stem cell factor or intrinsically produced stem cell factor as driving the proliferation of Schwann cells, we realized that other growth factor receptors such the PDGF receptor itself could also be involved. In addition, it became clear from the initial and subsequent work that understanding of intracellular pathways related to the overexpression of growth factor receptors was of paramount importance in understanding the hyperproliferative state characteristic of NF-derived Schwann cells.

More recent work concerning neurofibromatosis also supports this work. It has recently been reported that a deficiency of neurofibromin causes RAS-mediated hypersensitivity to a growth factor leading to chronic myeloid leukemia (16,17). The data supports the view that the response of the NF-derived Schwann cells to growth factors may be exacerbated due to chronic activation of RAS. Another interesting recent study demonstrated that mutant neurofibromin can interfere with the next step of RAS intracellular signaling, namely the association of RAS with Raf (18). Of considerable interest to our studies was the observation that although mutated neurofibromin would attenuate the stimulation of the mitogen-activated protein kinase by RAS, it could not influence the activation of this key intracellular enzyme by stimulation with platelet-derived growth factor. These results indicate that there are two separate pathways to the activation of MAP kinase. These two pathways are part of our overall model for "Pathways to Proliferation in Neurofibromatosis." The key role of activated RAS in the proliferative potential

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of Schwann cells was recently confirmed with a report from Nancy Ratner's lab that the ability of NF-1 deficient rat Schwann cells to hyperproliferate can be reverted by inhibiting the association of RAS with the membrane which is a key step in allowing activation of RAS (22). This report also demonstrated that Schwann cell hyperplasia can be induced by a kinase activated by cAMP (protein kinase A). The ability for the cells to hyperproliferate is depending on the extent to which neurofibromin is missing. In the cells which are homozygous there is an immediate hyperproliferative effect, whereas cells that our heterozygous become hyperproliferating after one day. An interesting observation was that prevention of RAS activation utilizing a farnesyl protein transferase inhibitor greatly diminished the ability of the null mutant cells to hyperproliferate. However, their potential for tissue invasion was not reduced (22).

The importance of cKit in NF is documented by two reports concerning individuals affected with both neurofibromatosis and a deficit in the expression of cKit, known as Piebaldism (19,20). These individuals were reported have absolutely no neurofibromas. This data supported our contention that cKit is involved in the etiology of the hyperproliferation characteristic of neurofibromatosis. When patients lack both neurofibromin and cKit, they also demonstrate a lack of hyperproliferation of Schwann cells. This is the *in vivo* counterpart of the *in vitro* experiments proposed as specific aim three in our original proposal. Coexpression of cKit and stem cell factor has also been demonstrated in breast carcinomas and evidence has been presented that the coexpression of the ligand and receptor could be responsible for growth deregulation in a significant number of breast carcinomas (21). Taken collectively, this data supports our hypothesis that the combined the combined affect of altered or absent neurofibromin expression and aberrant expression of the receptor KIT contribute to the Schwann cell hyperplasia which is characteristic of neurofibromatosis. It also provides the background to begin to look at intracellular signaling pathways which are related to abnormal growth factor receptor expression. This data also encouraged us to expand our investigations beyond the one cell line in which we had found the abnormal cKit.

BODY

This section of the progress report will include experimental methods, results, and discussion in relation to the statement of work outlined in the award proposal which contains three specific aims. Progress in achieving the specific aims and associated tasks, which were projected for completion in the first year, will be reported. In addition, we will document further research which has amplified and extended the original tasks. This additional research is entirely supportive of the original specific aims. The statement of work and specific tasks from the previous funded proposal are indicated below.

STATEMENT OF WORK

A. Specific Aim 1: effect of decreased neurofibromin expression on human Schwann cell proliferation
Task 1: third/fourth trimester of year 1, first trimester of year 2: Culture and expansion of human Schwann cells; determination of basal proliferation levels.
Task 2: first/second/third trimesters of year 2: Transfection of human Schwann cell with oligonucleotide antisense to neurofibromin; determination of the optimal conditions for viability and efficiency of transfection; Northern blot analysis; proliferation assay.

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B. Specific Aim 2: role of Kit expression in NF1-derived Schwann cell proliferation
Task 3: first trimester o year 1: Establishment of the cultures from frozen stocks. Verify basal doubling time.
Task 4: first/second trimesters of year 1: Determination of optimal conditions for transfection for each NF I -cell line; generation of clonal transfected cell lines.
Task 5: second/third trimester o of year 1: Collection of RNA for determination of DN-Kit transcript level of expression; proliferation assay.
Task 6: third trimester of year 1: DE Northern blot; quantitation of DN-Kit expression; correlative analyses DN-Kit expression/level of proliferation.

C. Specific Aim 3: effect of simultaneous Kit expression and neurofibromin alteration on human Schwann cell proliferation.
Task 7: fourth trimester of year 2: Determination of optimal condition for transfection of Kit constructs into human Schwann cells.
Task 8: first trimester of year 3: Establishment of clonal cells lines; determination of levels of Kit expression in various clones; proliferation assay.
Task 9: second/third trimesters of year 3: Transfection with antisense oligonucleotide against neurofibromin of Kit expressing human Schwann cells selected in Task 9, using optimal conditions determined in Task 2. Proliferation assay. Collection of RNA for Northern Blot.
Task 10: fourth trimester of year 3: Northern blot on double transfected Schwann cells. Correlative analysis level of Kit/neurofibromin expression and proliferation.

Please note that the tasks to be carried out in year one are only related to specific aim one and specific aim two. As outlined below, we have made significant progress in accomplishing these tasks; however, there have been some negative as well as positive findings in our studies. In order to continue to make good progress, we expanded on our research scope consistent with the continued aim of understanding molecular mechanisms of Schwann cell proliferation in NF-1. The organizing principle for our studies **"Pathways to proliferation in NF-1 cells"** is shown in Figure 1 which is included as a separate sheet on the next page.
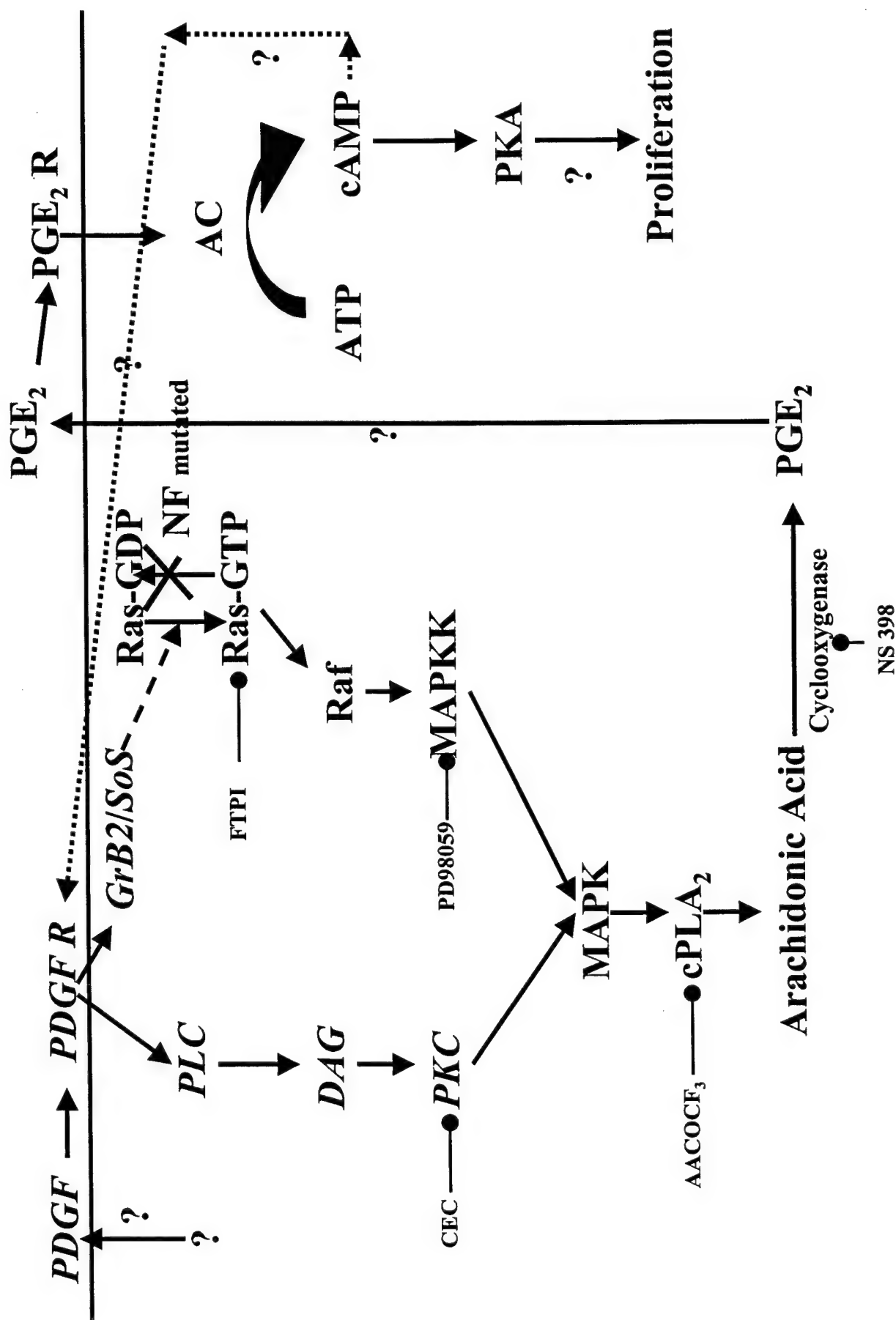
Figure 1. Pathway to proliferation in NF1 cells

A brief explanation of the relationship of this figure to our previously stated specific aims follows: The decision for a cell divide or not to divide is largely dictated by the state of activation of MAP kinase, shown as the key regulator in the scheme above. Note that there are two pathways by which MAP kinase can be activated within a cell. Both of these pathways are activated via growth factor receptors such as cKit or PDGFR. In particular, activation of these receptors leads to activation of phospholipase C which turn releases diacyl glycerol (DAG) which can then activate protein kinase C (PKC). Protein kinase C has been shown to phosphorylate and activate MAP kinase. Another pathway by which growth factor receptors activate MAP kinase is by RAS activation via the transducer molecules known as GrB2/SoS. These transducer molecules are activated by the growth factor receptors and in turn activate RAS. The activated RAS then combines with Raf, which subsequently activates MAP kinase kinase leading to the activation of MAP kinase itself. Other aspects of this pathway will be discussed later, but of particular interest to the growth factor receptor expression, the topic of this proposal, note that elevation of cAMP is known to lead to further expression of growth factor receptors such as cKit and PDGFR. Thus, this scheme provides a working hypothesis by which to link the overexpression of growth factor receptors and proliferation mediated via MAP kinase.

Our investigations from the initial funding of this work to the present have resulted in the publications of three full length papers (included as appendices to this progress report) which will be discussed with respect to the specific aims and progress which we are making in that regard. The three full-length papers and nine abstracts generated from the first year of research are listed below.

Papers Published:

1. Lopez, T. and G.H. DeVries (1999) Isolation and culture of primary Schwann cells from fetal human peripheral nerve. *Exp. Neurol.,* 158(1):1-8.

2. Badache, Ali, N. Muja, and G.H. DeVries (1998) Expression of Kit in neurofibromin-deficient Schwann cells: role in Schwann cell hyperplasia associated with Type 1 Neurofibromatosis. *Oncogene,* 17:795-800.

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9.   Lopez, T.J., M.F. Dauzvardis, and G.H. DeVries (1996) Purification and Functional Analysis of Primary Fetal Human Schwann Cells. *Soc. Neurosci. Abst.*, 22:1725.

Results with Respect to Specific Aim One: Effect of Decreased Neurofibromin on Human Schwann Cell Proliferation

Note that the major task (Task 1) to be accomplished with respect to this specific aim was to culture and expand human Schwann cells and determine their basal proliferation levels. The major accomplishments with respect to this task are documented in the paper, "Isolation in Serum Free Culture Primary Schwann Cells from Human Fetal Peripheral Nerve" by Lopez and De Vries which is included as Appendix 1 in this progress report. In order to facilitate the culture of human Schwann cells, we decided to initially utilize human fetal material since fetal tissue is much easier to dissociate and there are fewer problems with fibroblast contamination. In addition, we established a liaison with the University of Maryland tissue bank from which we could reliably obtain human fetal nerves for culture. We then devised a method for isolating Schwann cells from human fetal peripheral nerve and maintaining cultured Schwann cells *in vitro* under serum-free conditions. The final cell preparation consists essentially of pure Schwann cells which have a bipolar, spindle-shaped morphology. They align themselves in fascicles and express typical glial cell markers. Utilizing the neuregulin growth factor NDF-beta, the Schwann cells can be maintained in serum-free conditions for several months. When cocultured with neurites, the Schwann cells strongly associate with the neurites as demonstrated on the cover photo on the *Experimental Neurology* included with the reprint. In this photomicrograph, the alignment of the rhodamine-labeled S-100 positive Schwann cells with the fluorescein-labeled neurofilament positive neurites is very evident. As noted later in this report, these cell preparations were utilized as controls in several experiments to establish basal levels of metabolites related to Schwann cell proliferation.

Next, we made attempts to immortalize the primary Schwann cells using a retrovirus infection technique and a construct termed "mycER" (See Abstract 6). The methodology used in this approach is illustrated in the transformation strategy cartoon demonstrated below.
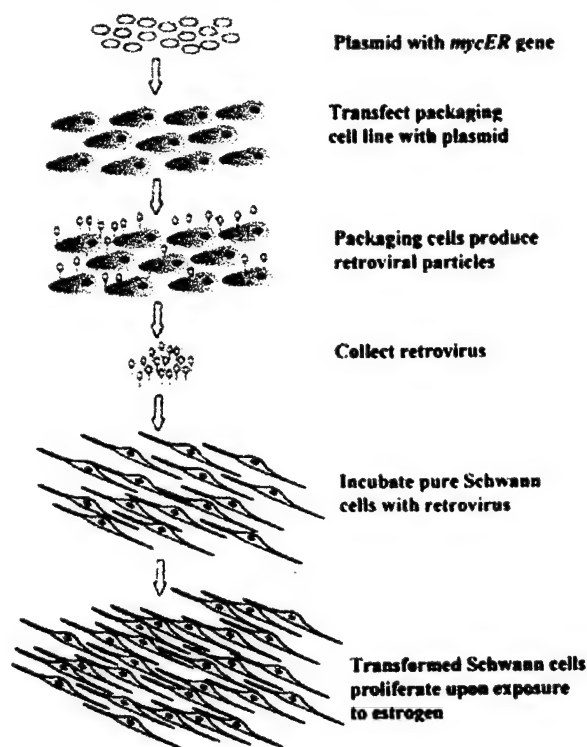

Fig 2 - Viral particle transfection protocol

The principle of this method involves successful integration of the plasmid into the genome of the host cell, followed by exposure of the cell to estrogen which causes the mycER product containing the gene of interest (in this case c-myc) to translocate to the nucleus where it will activate gene transcription. This strategy was used to transfect the human fetal Schwann cells. In order to validate the effect of estrogen on the transfected cells, we compared the proliferation rate in the presence or absence of estrogen, as shown in the figure 3 below.
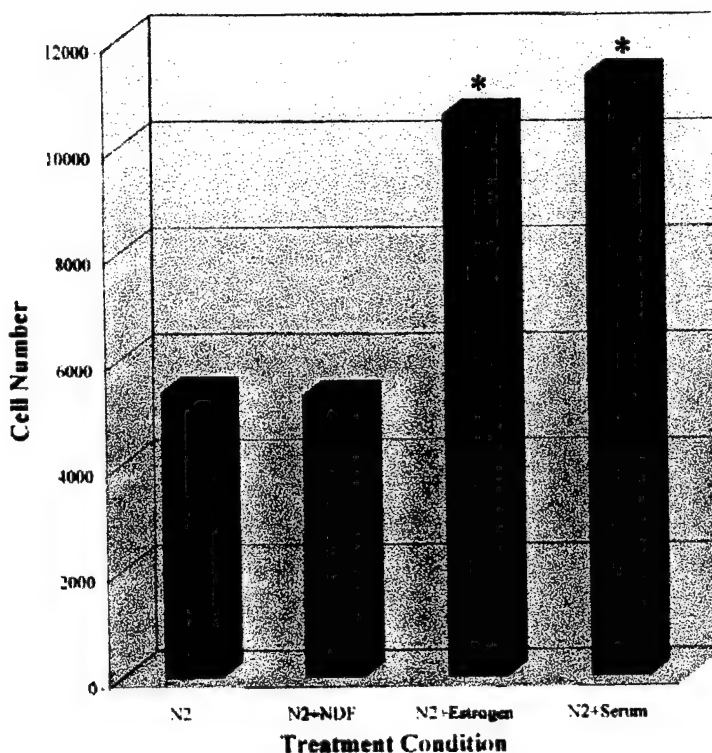
Figure 3- Effect of estrogen on proliferation of transfected fetal human Schwann cells

The following experimental methods were used in this study which is documented in Abstract 4 included in the appendix. .

Proliferation Assays

Proliferation was determined using Hoechst dye staining of cells (23) and a Cytofluor Multiwell Plate Reader Series 4000 (Perspective Biosystems). To generate a standard curve (Fig. 3), known numbers of SC were plated in Falcon 96 well microtitre plates, and allowed to settle. Wells were washed, filled with 100 µl sterile water, and incubated at 37'C, frozen at -80'C for 45 minutes, and thawed. An equal volume of Hoechst dye solution (20µg/ml in 10mM Tris, 2M NaCl, ImM EDTA, and 2mM Na azide) was added to each well, and the plate was 'incubated at room temperature for 10 minutes. Fluorescence readings were taken at 360nm excitation and 460nm emission. For experimental protocols, 5000 cells per well were treated with different culture conditions, with the culture medium volume held at 100 µl and changed every three days. Cell number was determined as above.

pMVmcyER Retroviral Vector Construct

The chimaeric gene mycER consisted of the human c-myc gene fused to a fragment of the cDNA encoding the hormone-binding domain of the human estrogen receptor (24). The plasmid pMV-7 was

created as a derivative of pMV (using the parent plasmid p 10 1 A 0 1, a pBR322 derivative containing the complete Moloney murine sarcoma virus) to contain a deletion mutant of the Moloney murine sarcoma virus (MoMSV; 25). Like pMV, pMV7 has MoMSV 5' and 3' LTRs intact, but also contains the neomycin phosphotransferase gene driven by the herpes simplex virus thymidine kinase (P_{tk}) promoter (P_{tk}neo) small sequences adjacent to LTRs, and amp' external to proviral sequences. however a 3.95 kb fragment containing most of the viral genome has been excised from between the LTRs. The chimaeric m cER gene sequence was inserted into the site of the viral genome deletion as an EcoR1 fragment, and the plasmid designated pMVmycER (24). (Plasmid pMVmycER was a generous gift of M. Bishop; permission for use of c-myc from M. Bishop and of estrogen receptor from P. Chambon).

The HB 10 1 strain of E. coli was transfected with pMVmycER, and plasmid minipreps were performed to collect microgram quantities of pMVmycER from bacterial preparations.

Retroviral Packaging

The amphotropic PA317 packaging cell line (ATCQ harbors mutant retroviral packaging construct DNA helper virus, however mutations prevent helper virus production and transfer of packaging function. Further, there is no detectable recombination between helper virus and proviral-containing plasmids such as pMVmycER, and PA317 produce high titres of retrovirus (26). The PA317 cell line was grown to subconfluence (70%) in culture medium (DMEM with high glucose, Gibco, fetal bovine serum {FBS), HyClone, 100U penicillin and 100 µg/ml streptomycin (Gibco) prior to transformation with pMV mycER.

Plasmid pMVmycER DNA collected from minipreps was quantified spectrophotometrically, and an appropriate quantity of pMV mycER was added to the Superfect Transformation Reagent (Qiagen) according to manufacturer's instructions. PA317 cells were transformed using the plasmid pMVmycER and the Superfect Kit in the following manner. PA317 cells were washed three times with sterile phosphate buffered saline, and exposed to plasmidcontaining Superfect reagent for two hours. Immediately following two-hour exposure, the transfection reagent was aspirated off of the cell cultures, cultures were again washed three times with sterile PBS, and fresh culture medium was added to each culture dish. Transfected PA317 cells were grown for two days, and selection with neomycin sulfate began after 48 hours. Following selection, transfected cells were grown to 80% confluence and fresh culture medium without neomycin was added. Conditioned medium containing retroviral particles was collected 24 hours after culture in neomycin-free medium.

Cells transformed with the mycER construct were treated as shown beneath each bar and the rate of proliferation was quantitated by utilizing Hoechst dye staining of cells and a Cytoflour Multiwell plate reader. The cells were allowed to proliferate for 3 days at which time the florescence reading (which was shown to be linearly related to cell number) was obtained.

Note that in the basal conditions, in the presence of serum-free media, N2 or N2+ the NDF growth factor, there was no mitogenic response. However, addition of estrogen, caused an approximate doubling of cell number indicating an affect of the transformation on the ability of the cells to expand. However, we were disappointed to observe that serum caused a similar effect. Note that the cells treated with serum proliferated almost as much as those treated with estrogen. It is possible and likely that estrogen in the serum was responsible for this proliferative effect.

This data demonstrated that there would be limited utility to this procedure since any assay done in the presence of serum would run the risk of causing the cells to proliferate. Therefore, no further attempts were made to utilize this methodology to immortalize cells. Other approaches are being considered, including the commercially available inducible mammalian expression system, which is available from Strategene. In this regard, a former student, who is now a postdoctoral fellow at the Medical College of Virginia, is providing advice for the use of this system which he has already successfully used. In essence, this system is a gene transfer system that allows precise control of gene expression in a wide variety of mammalian cell types. The system is based on the finding that the insect hormone ecdysone, or its analogue ponasterone A, can activate transcription to mammalian cells which express the ecdysone receptor and a promoter containing a binding site for the ecdysone receptor. This system would get around the problem of having an inducer present in serum since pontasterone A would never be present in any media utilized to study the human Schwann cells. This system has been successfully used in a number of different applications. It is usual to obtain a thousand fold induction of a reported gene with negligible basal expression utilizing this system. We are hopeful that this will obviate some of the problems previously encountered with the mycER system.

Obviously, since we were not successful in our attempts thus far to culture and expand human Schwann cells, it was not possible to determine the basal proliferation levels as outlined in the statement of work. We will continue to pursue this aim utilizing not only the fetal human Schwann cells, but the more relevant adult human Schwann cells obtained from human nerve biopsies.

Results with Respect to Specific Aim Two:

This specific aim which is related to the role of KIT expression in NF-1 derived Schwann cell proliferation, consists of 4 specific tasks and was proposed for completion during the course of year one. Some of the specific tasks have been accomplished and others were not possible to complete due to technical difficulties outlined below.

With respect to task three, the establishment of the cultures from frozen stocks and verification of basal doubling time, we have been successful in obtaining this data as shown in Table 1 below.

Table 1 – Basal doubling times of Schwann cell lines

CELL LINE	DOUBLING TIME (hours)
ST88-14	48.7
T265-2C	40.7
STS26T	66.3
NFIT	81.0

In this experiment, cells were plated at a density of 10,000 cells per well in a 96 well plate and allowed to divide for fixed amounts of time (24,48 and 72 hrs) after which cell number was determined as previously described (28). Doubling time was then determined by regression analysis.

In addition to establishing the basal doubling time of these cell lines, we have obtained two other cell lines from Dr. Jeff DeClue at the National Institute of Health. These cell lines are termed NF90-8 and NF88-3 and are derived from NF-1 patients. The NF90-8 cells are currently being cultured in the lab and their doubling time being established. We have had trouble establishing the NF88-3 and find that they are dividing very slowly and have not remained viable under our present culture conditions. We are hopeful that manipulation of the growth conditions will allow their success for growth. However, both of these cells are derived from neurofibromatosis patients and will be extremely useful in validating the statistical significance of our findings with respect to molecular mechanisms of proliferation in Schwann cells derived from NF patients.

Task four involved determination of optimal conditions for transfection for each NF-1 cell line and generation of clonal transfected cell lines. We refer to the transfection system which is illustrated in the cartoon shown in Figure 2. Utilizing the methodology of Perkins et al (27), we have successfully employed a very efficient system for transfecting cells in culture. This involves transfecting plasmids into a packaging cell line. The packaging cell contains only

part of the information required to package and secrete viral particles. The missing information for packaging is supplied by the plasmid containing the gene of interest. Therefore, cells which are transfected begin secreting virus. The virus in turn can infect other packaging cells and further amplify viral production. The retrovirus then can be collected from the supernatant and added to cultured cells of any kind, which will resist in the incorporation of the plasmid into the genome of the cultured cell. The previously mentioned inducible mammalian expression system requires several transfections and we will use this methodology to carry out the transfections. Establishment of this methodology in combination with the mammalian inducible cell line has enabled us to more effectively introduce genes of interest into both NF-derived and normal human Schwann cells.

With respect to task five, which involved collection of RNA for determination of dominant negative KIT transcript levels of expression and carrying out a proliferation assay, the following has been accomplished. With respect to the proliferation assay, we have obtained a Cytoflour Fluorescent Plate Reader which greatly increases the sensitivity of our proliferation assays for Schwann cells. Previous work utilized tritiated thymidine and involved generation of radioactive waste. The present method obviates this problem. The basis for this fluorescent-based proliferation assay is a proprietary green fluorescent dye termed CyQUANT GR dye, which exhibits strong fluorescence enhancement when bound to cellular nucleic acids. At the end of a proliferation assay, cells are simply frozen in their 96 well plate, lysed by an addition of a buffer containing the dye. The fluorescence is then measured directly. There are no washing steps, growth medium changes, or long incubations. This assay has been used for a number of different cell types and is sensitive and specific for proliferation. Therefore, with respect to the proliferation assay, we have successfully established sensitive and specific assay conditions for our studies. A dose response curve for this method is shown in figure 4 below.

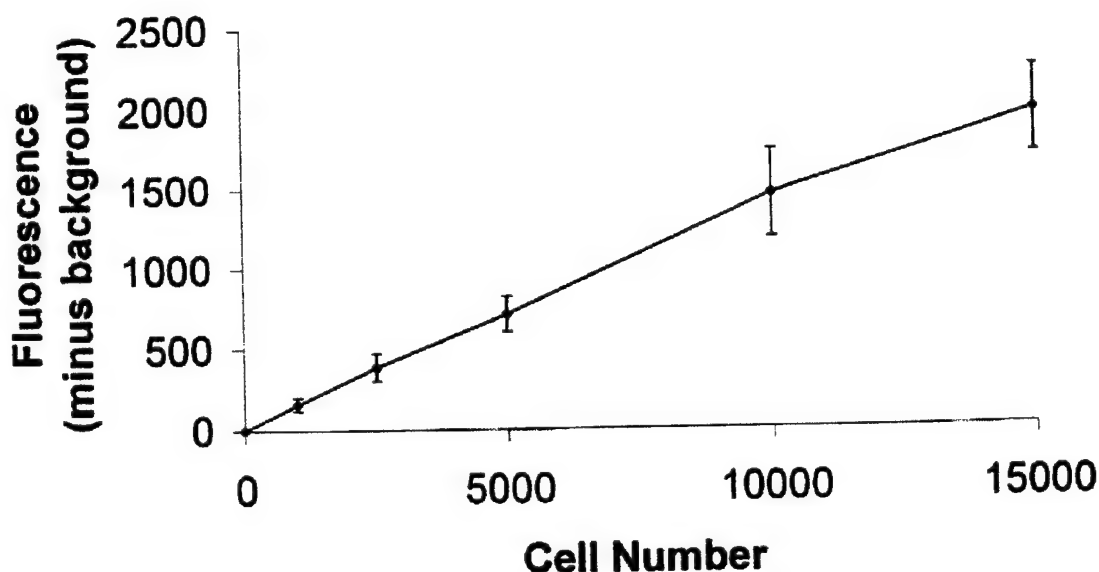## Cell Proliferation Assay
Schwann Cells

Figure 4- Effect of cell number on fluorescence yield in cell proliferation assay.

Note that there is excellent linearity up to 15,000 cells per well and that the lower limits of sensitivity are at approximately 500 cells per well. Utilization of this method for a proliferation assay in which axolemma enriched fraction (AEF) which is utilized as a mitogen (28) is shown in the Figure 5 below.
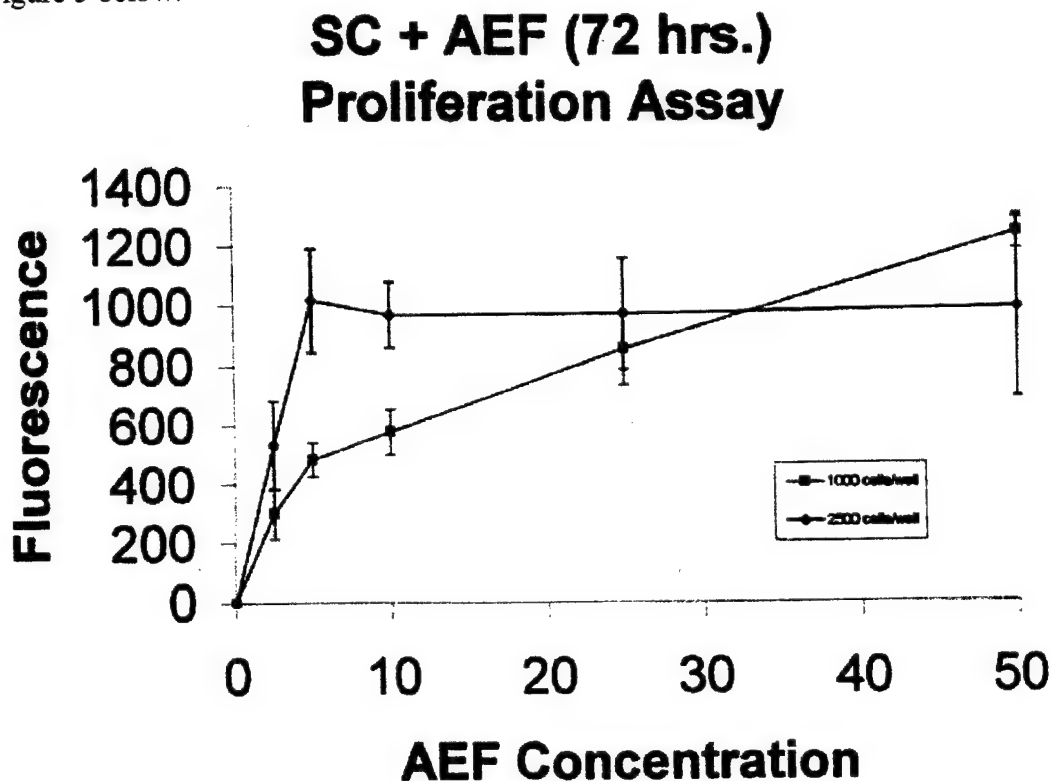
SC + AEF (72 hrs.)
Proliferation Assay

Figure 5- Validation of Cytofluor proliferation assay. See text for experimental details.

In this experiment, either 1,000 cells per well or 2,500 cells per well were stimulated with the inducted concentrations of axolemma for 72 hours and the fluorescence related to cell number was determined. Note in both cases, there was an excellent dose-dependent response to axolemma which peaked rapidly in the case of 2,500 cells at less than 10 micrograms of axolemma. In the case of 1,000 cells per well, the rate of increase was rapid at first but more gradual later on in the dose response curve.

With respect to experiments involving the dominant negative KIT transcript, we have had a number of technical problems. We obtained the dominant negative KIT transcript from Dr. G. Krystal. He had successfully utilized this construct in his work with the breast carcinoma cell lines (29). Prior to using these constructs extensively, we decided to restriction digest and sequence parts of the construct in order to be sure that we had obtained the correct construct. To our chagrin, we found out that the construct labeled as the dominant negative cKit construct was in fact a normal full length cKit clone. This confusion created considerable delay in getting these experiments under way. We have now identified, through sequencing, the correct construct

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which had been labeled as a full length cKit construct. We plan to use this dominant negative construct and incorporate it into the proper plasmid for being transfected with a packaging cell line so that we can successfully carry out this part of the statement of work.

Since we were not able to identify the correct dominant negative KIT vector, we could not obviously carry out tasks 5 and 6. However, with the availability of the effective transfection strategy utilizing packaging cell lines and with the subsequent identification of the correct construct, we expect to be able to carry out these tasks in the near future.

In the face of technical difficulties with the dominant negative KIT construct being utilized to lower KIT expression, we decided to use the antisense to KIT approach to accomplish the same goal.

Use of antisense oligonucleotides to suppress c-kit expression in ST88 and T265 cells.

In the original proposal we postulated that the abnormal proliferation in NF Schwann cells is due in part to the high expression of c-kit, and that decreasing the cellular levels of c-kit would direct the cells towards a normal level of proliferation. Our efforts to use antisense methodology to reduce the expression of c-kit in NF cell lines expressing abnormally high levels of the receptor have been unsuccessful thus far. We began by first incubating unmodified 21-mer c-kit sense or antisense oligonucleotides with the cells and determined the effect of this treatment on levels of c-kit protein. Yamanishi et al used this approach to show that c-kit antisense could specifically inhibit the growth of small-cell lung cancer cells (30). For our experiments, ST88 or T265 cells were seeded in six well plates at a density of 10^5 cells/well and incubated in medium (1ml/well) containing 20 µg/ml of either sense (5′-ATGAGAGGCGCTCGCGGCGCC-3′) or antisense (5′-GGCGCCGCGAGCGCCTCTCAT-3′) oligonucleotides. The initial dose was supplemented with an additional 10 µg at 24 and 48 h. Five days after the initial treatment, the cells were harvested and processed for immunoblot analysis of c-kit protein levels. Figure 6 shows the levels of c-kit expression in control cells and cells treated with sense or antisense oligonucleotides for five days.
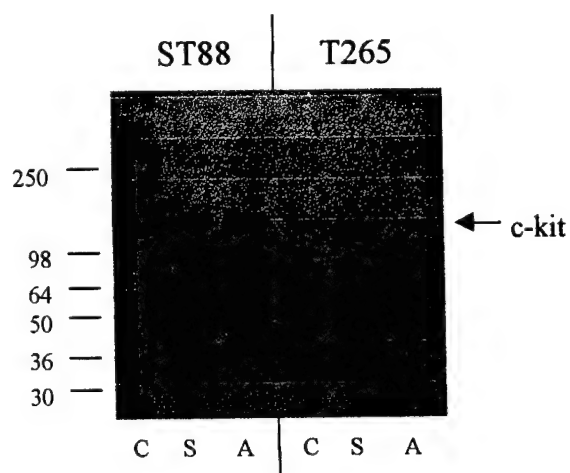

Figure 6 Immunoblot analysis of c-kit expression in NF cell lines after treatment for 72 h with c-kit sense (S) or antisense (A) oligonucleotides. C-kit immunoreactivity from the cells treated with antisense showed no reduction when compared to sense-treated cells. (C) = untreated control cells.

Under these conditions, the presence of c-kit antisense in the incubation medium did not reduce the levels of c-kit in either cell line when compared to the same cells incubated with sense

oligonucleotides (Figure 4). One possible and likely explanation for these results is that there was insufficient uptake of the oligonucleotides to inhibit the expression of the c-kit gene. To increase uptake efficiency, we incubated the oligonucleotides with Lipofectamine reagent (Gibco) according to the manufacturer's instructions before adding them to the cultures. This reagent is commonly used to increase transfection efficiency, and we have used this in the past for effectively introducing oligonucleotides into cultured oligodendrocytes (31). Different amounts of Lipofectamine were used initially in order to find an optimal concentration. It was found that Lipofectamine at concentrations greater than 25 µl/ml resulted in cells detaching from the dish, suggesting a degree of toxicity. Therefore, we used Lipofectamine at concentrations at or below 20 µl/ml and tested for its effect on oligonucleotide uptake by the NF cells.

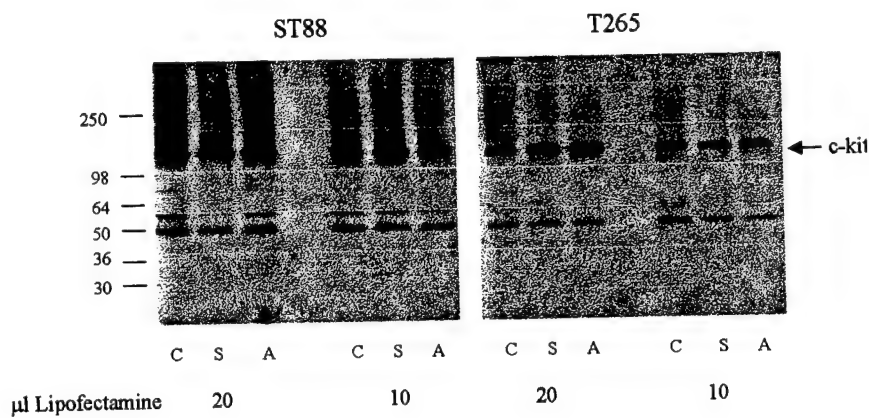**Figure 7.** Immunoblot showing c-kit expression in NF cell lines after incubating for 24 h with 20 µg/ml sense (S) or antisense (A) oligonucleotides and either 20 or 10 µl/ml Lipofectamine. (C) = untreated control cells

Figure 7 shows immunoblots from cells incubated 24 h with 20 µg of either sense or antisense oligonucleotides in the presence of 10 or 20 µl/ml Lipofectamine. Only the ST88 cells incubated with antisense oligonucleotides and 10 µl/ml Lipofectamine demonstrated a possible decrease in c-kit immunoreactivity when compared to cells treated with sense oligonucleotides (Figure 5; first panel). However, the level of staining for the antisense-treated cells was similar to that of untreated control cells. The possibility that Lipofectamine at a concentration of 10 µl/ml could increase the efficiency of oligonucleotide uptake by the NF cells was examined further by determining the effect of the oligonucleotide/ Lipofectamine mixture on c-kit expression over a longer time period. Cells were incubated with 20 µg sense or antisense oligonucleotides/10 µl LipofectAMINE™ and collected 24 and 48 h to determine whether increasing the length of incubation would increase any inhibitory effect on c-kit expression.
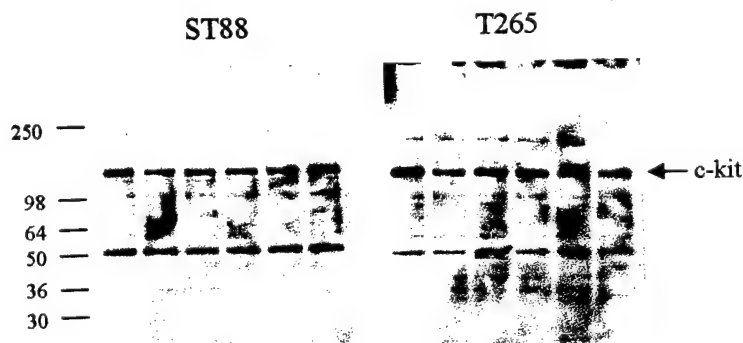

Figure 8. Immunoblot showing the levels of c-kit protein expressed by NF cells incubated for 24 or 48 h with sense (S) or antisense (A) oligonucleotides to c-kit and 10 µl Lipofectamine. (C) = untreated control cells

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Figure 8 shows the results from this experiment. For both time periods, the antisense-treated cells showed no decrease in c-kit protein expression relative to the sense-treated cells.

A second approach that we are using to increase uptake of the oligonucleotides by the cells is to incubate the cells for a brief time with sense or antisense oligonucleotides in the presence of 0.2 U/ml streptolysin O. Streptolysin is a pore-forming agent that allows the cell membrane to become permeable to oligonucleotides with essentially no toxicity (32). Initially we tested whether the NF cells would survive streptolysin treatment by incubating T265 and ST88 cells with 20 µg/ml sense or antisense oligonucleotides in the presence of 0.2 U/ml streptolysin for 5 minutes. The cells were allowed to incubate for an additional 18 h before harvesting. Figure 9 (below) shows the level of c-kit immunoreactivity on immunoblots from cells treated in this manner. Only the T265 cells showed a possible reduction in levels of c-kit protein with antisense treatment.
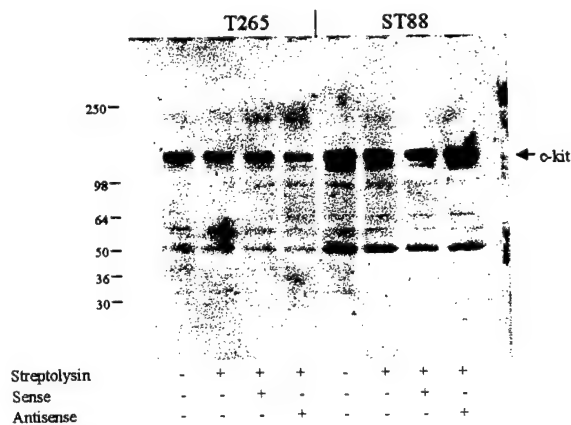

Figure 9. Immunoblot showing the levels of c-kit protein expressed by NF cells after introduction of c-kit sense or antisense oligonucleotides into the cells by streptolysin treatment. Cells were treated with streptolysin (0.2 U/ml) and oligonucleotides (20 µg/ml) for 5 minutes and then incubated for and additional 18 h.

Since introduction of oligonucleotides into cells by this method is dependent on both time and oligonucleotide concentration (Barry, et al., 1993), further experiments are being carried out with this technique in an effort to optimize the inhibition of c-kit expression.

In summary, the antisense approach to lowering cKit expression is somewhat promising but needs to be maximized in order to achieve significant lowering of the expression of cKit.

Results with Respect to Specific Aim Three:
This task involves the effective simultaneous upregulation of KIT expression and down regulation of neurofibromin on human Schwann cell proliferation. The tasks associated with this

specific aim are not to be carried out until later in the course of the proposal beginning with the fourth trimester of year two.

Work Accomplished Directly Related to Specific Aim Two, But Not Specifically Stated as a Task in the Initial Proposal:

The overall goal of this specific aim was to understand how KIT expression was related to Schwann cell proliferation in neurofibromatosis. In the case of many cancerous conditions, a gene important in early development is inappropriately reexpressed when the cancer develops. In order to investigate a potential development for cKit, we carried out the following experiment. Sciatic nerves were dissected from rat pups at the following ages: newborn (0), 2 days, 5 days, 7 days, 10 days, and 15 days. In addition, we also obtained sciatic nerve from adult rats. A 10% homogenate was made of the sciatic nerves in phosphate buffered saline. Protein was determined using the Hartree assay (43) and the protein was solubilized and subjected to polyacrylamide gel electrophoresis as previously described (33). A profile of the resulting gels is shown in Figure 10 below.
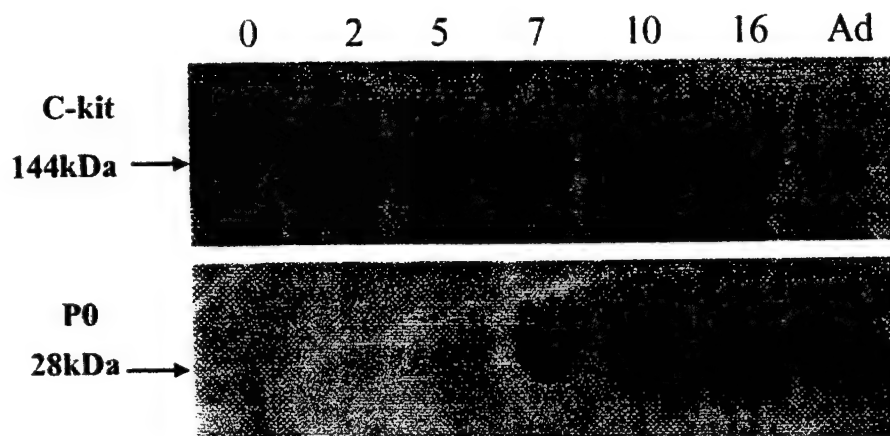

Figure 10- Western blot analysis of P0 and c-Kit in developing rat sciatic nerve. The numbers above each lane indicate the postnatal age of the nerve homogenate which was analyzed by Western blotting.

Note the inverse relationship between the expression of cKit and Po. Po is the major glycoprotein of peripheral myelin and is closely related to differentiation. It is known that Po is only expressed in Schwann cells, therefore this is a good marker for the differentiation of these cells. Po only begins to be present as myelination reaches significant proportions in peripheral nerve beginning at approximately day five and continuing on through day sixteen. Note that the opposite is the case with the cKit expression which is highest in the newborn animals and with development gradually tapers off. The bands were quantitated by densitometry using a Biorad Model 65670 Densitometer and the results are shown in Figure 11.

C-kit and P0 Are Inversely Regulated During Development
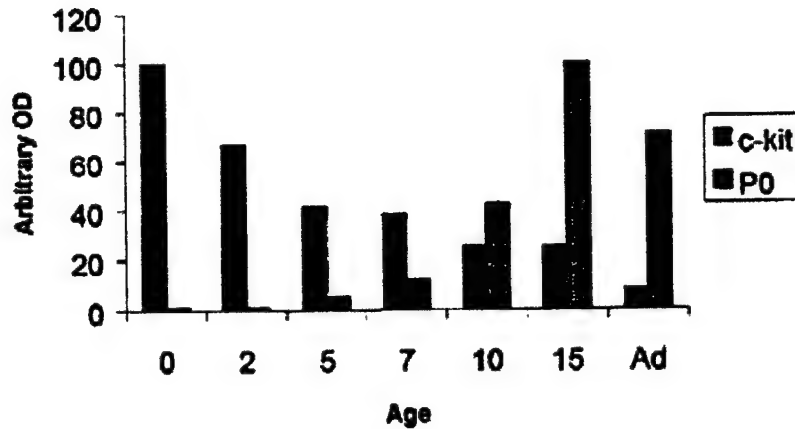

Figure 11- Densitometric analysis of P0 and c-kit in Western blots of developing sciatic nerve.

Note the clear inverse relationship between the development of peripheral nerve in which Po expression increases and cKit in which expression is much higher in the newborn animals than in adults where it is almost negligible. Obviously, cKit could be expressed by a number of different cells types in peripheral nerves that were analyzed. Therefore, we evaluated the various cellular constituents of peripheral nerve for the expression of cKit. The results of this investigation are shown in Figure 12.
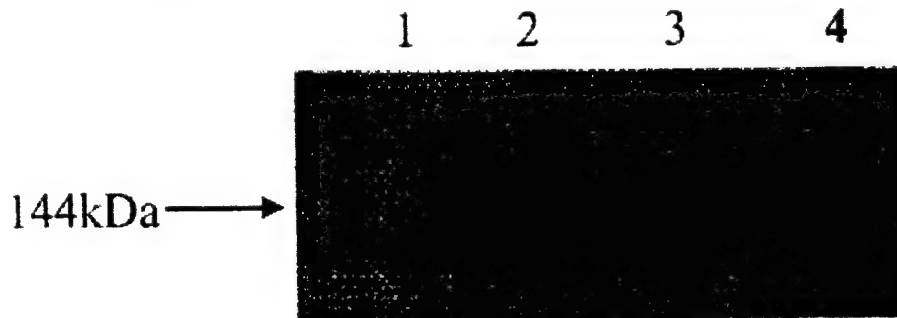

Figure 12- Western blot analysis of c-kit in cellular elements of rat sciatic nerve.
Lane 1=fibroblasts, lane 2=HEL cells, lane 3=ST-88-14 cells, lane 4= Schwann cells.

Note that rat fibroblasts, cultured from an explant of minced rat skin, are virtually lacking expression of cKit. HEL cells, shown in lane two, are a positive cell control in which cKit is known to be expressed in high quantities. The ST-88 cells (lane 3) expressed high levels of KIT as we have previously documented (33). Primary Schwann cells (lane 4), on the other hand, compared to fibroblasts, are the only cell type which express significant amounts of cKit. Therefore, we believe that our analysis of cKit in whole nerves is an accurate reflection of cKit being present in the Schwann cells and not fibroblasts and reflects a role for this molecule during

early development. In order to further ascertain the presence of KIT in neonatal rat Schwann cells, we carried out a RT PCR amplification of mRNA obtained from neonatal rat Schwann cells. The results are shown in the Figure 13 below.
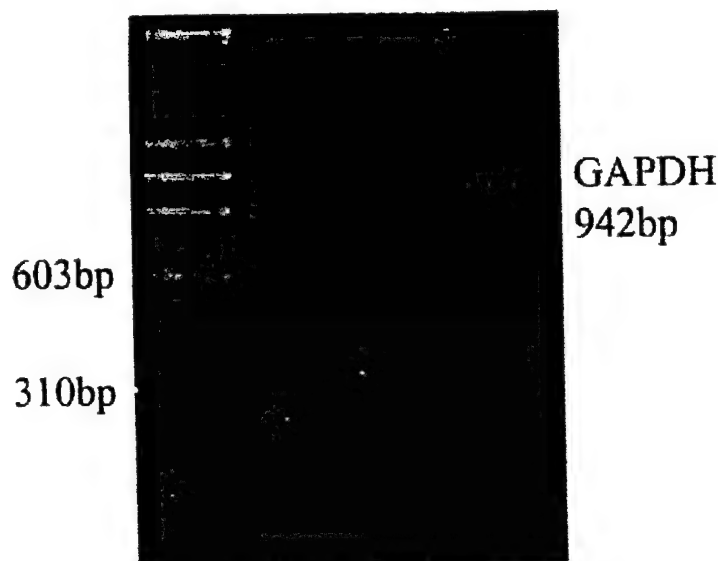

Figure 13- Reverse transcription-polymerase chain reaction (RT/PCR) amplification for c-kit and GAPDH in cultured neonatal Schwann cells.

Note the prominent presence of the expected size transcript (513 base pairs) in the central lanes of this figure. In order to further ascertain that this was indeed the authentic cKit, we sequenced the 513 base pair transcript obtained by RTPCR. The transcript was sequenced in the 5` to 3` direction as well as the 3` to the 5` direction. Comparison of the sequence obtained to the authentic rat cKit sequence revealed complete identity for at least these 513 pairs indicating that the cKit found in the neonatal rat Schwann cells was indeed authentic (data not shown). These results give evidence that cKit does play an important role in Schwann cells early in development. The question remained what was the function of the cKit expression during early development. In order to evaluate this, the following experiment was carried out. Stem cell factor, the ligand for cKit and was obtained from Amgen. The dose-dependent effect of stem cell factor on the ability of neonatal Schwann cells (known to contain significant amounts of the cKit receptor) was evaluated. The results are shown in Figure 14 below.
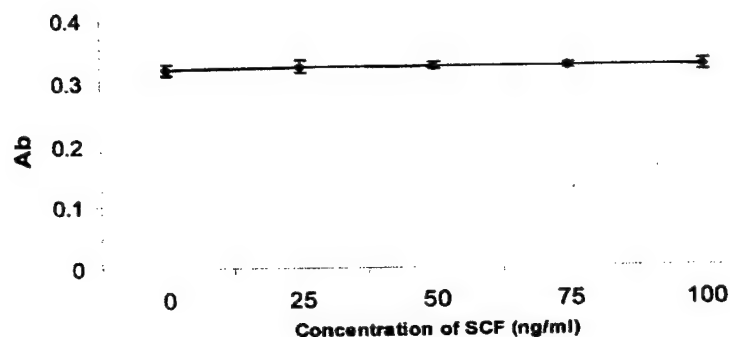
Figure 14- SCF is not a mitogen for neonatal Schwann cells. Increasing amounts of rodent SCF was added to cultured Schwann cells followed by the MTT assay for proliferation (33).

Note that there was absolutely no dose-dependent increase in the number of Schwann cells as would be evident by an increase in absorption in this colorimetric MTT assay, which was carried out after three days of continuous stimulation in culture. Therefore, although it is certain that the cKit expression in Schwann cells is an early developmental event and developmentally regulated in an inverse way with respect to differentiation, the exact role of cKit is still not clear. We next investigated whether or not stem cell factor could potentiate the mitogenic effect of NDF-B (the beta form of NDF). The results are shown in Figure 15 below.
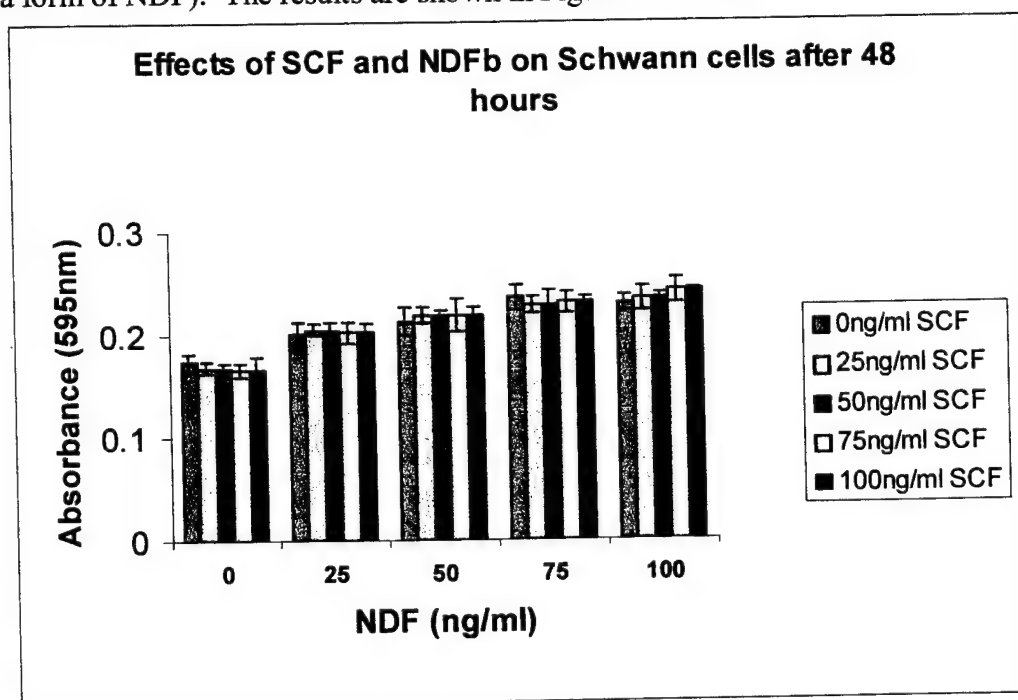

Figure 15- SCF does not potentiate the mitogenicity of NDF. SCF was added in increasing concentrations as indicated followed by the MTT proliferation assay (33).

Note the lack of any synergistic effect of SCF on NDF-simulated proliferation. We concluded that neither SCF nor KIT were not strong mitogens, nor did SCF act in a synergistic way with NDF-B.

The paper, "Expression of KIT in neurofibromin deficient human Schwann cells: role in Schwann cell hyperplasia associated with type 1 neurofibromatosis," included as Appendix 2 to this progress report and follows up our initial observation of overexpression of cKit in the ST88 cell line. In order to make a case that the cKit overexpression is related in some way to neurofibromatosis, we felt it was important to establish correlation between the lack of neurofibromin in a given cell line and the presence of overexpression of cKit. Indeed, this proved to be the case as demonstrated in Figure 1 of the paper. Analysis three neurofibroma derived cell lines for cKit and a Schwannoma cell line, STS26T, for expression of neurofibromin in cKit, revealed that only cells lacking neurofibromin (i.e. ST88-14 and T265-2C) overexpressed cKit. The NF-1T cell line as well as the STS26T both contained normal levels of neurofibromin and did not overexpress cKit receptor. Therefore, there must be some cogent way, based on intracellular signaling mechanisms, to explain how the lack of neurofibromin leads to overexpression of receptors. In addition, we also reported the coexpression of cKit in spindle shaped Schwann cells in a tumor derived from a NF patient. cKit positive round cells were also noted, which most likely were mast cells. We also demonstrated that proliferation of the NF-derived Schwann cells was related to growth factor receptor activated tyrosine kinase. In a dose dependent manner, tyrosine kinase inhibitors such as genistein or tyrphostin caused inhibition of proliferation in these Schwann cells. To further investigate the potential of an autocrine loop involving stem cell factor and cKit receptor as being responsible for cell proliferation, we incubated two of the NF-derived cell lines with antibodies to stem cell factor. We found that there was no dose dependent decrease in proliferation as a result of this treatment.

This data indicated that although cKit was overexpressed in these cells, it was not being activated by exogenously produced stem cell factor to create an autocrine loop which stimulated proliferation. In a similar way, if we inhibited the cells with an antibody directed against KIT, there was no change in the growth of the NF-derived cell lines. Furthermore, suramin, an inhibitor of autocrine loops involving PDGF type receptors, did not cause any significant change in the proliferation of the NF-derived cell lines. However, it was interesting to note that the two cell lines which overexpressed cKit receptor ST88-14 and T265TC, could respond to exogenously added stem cell factor in a dose dependent manner increasing their rate of proliferation. However, other cells such as NF-1 T and STS26T, not lacking neurofibromin, did not significantly respond to this factor. We concluded that although cKit receptors were overexpressed, there was not an autocrine loop which was responsible for the basal level of proliferation which these cells demonstrated in culture.

Next, we wondered if cKit overexpression in these lines was unique or whether other receptors in the same tyrosine kinase family were also overexpressed. Therefore, in the second paper, "Neurofibrosarcoma derived Schwann cells overexpressed platelet derived growth factor (PDGF) receptors and are induced to proliferate by PDGFBB" (included as Appendix 3), we investigated the potential overexpression of PDGF receptors in NF-derived Schwann cell lines. To our surprise, we found that in the cells lacking neurofibromin, PDGF BB was a potent mitogen. Even though the cells were already dividing at an appreciable rate, this rate could be substantially increased in a dose and time dependent manner by the growth factor PDGFBB. This was not the case for the STS26T or for a transformed Schwann cell line known as RSC96.

FGF2 (basic FGF) also caused a modest increase in the proliferative rate. It was interesting to note that NDF-B was virtually without effect on proliferation even though this mitogen is known to be the most potent mitogen for Schwann cells. In any Schwann cells, mitogens require elevation of intracellular cAMP for maximal activity. We used the drug forskolin to elevate cAMP (34). When we investigated the extent to which forskolin could potentiate the mitotic response of these cells to PDGF we found no change in the proliferation rate. This data implied that the NF-derived Schwann cells had already elevated their intracellular cAMP levels, a hypothesis which our preliminary data has now substantiated. Next, we investigated in detail the expression of PDGF receptors in the various Schwann cell lines. We found that the NF containing Schwann cell lines (STS26T, T265TC) did not express PDGF receptor. However, in the case of the NF-derived Schwann cell lines, there was abundant expression of both the alpha and beta isoforms of the PDGF receptor. We also investigated the time course of the PDGF BB induced activation of PDGF receptors. We found, similar to what others have reported, that there was a rapid phosphorylation of a protein having a molecular weight consistent with and identical to that of the PDGF receptors. We also demonstrated that both the alpha and beta receptor were present during the period of the time course of the experiment. As expected, only the BB isoform of PDGF was effective as a mitogen for the NF-derived Schwann cell lines. Surprisingly we found that only the PDGF B receptor could be activated significantly by PDGF BB. The alpha receptor, although present at substantial levels, was not activated by either PDGF AA or PDGF BB. The significance of this finding is still uncertain.

Neuregulins are among the most potent mitogens known for Schwann cells. A most interesting finding (see Figure 1) was that all of the cell lines lack the ERB B3 receptor which is absolutely required for the mitogenic activity of neuregulins. In addition, the STS26T was lacking the ERB B2 receptor, although this receptor was present in both the neurofibromin containing and the neurofibromin lacking cell lines. However, it should be noted that ERB B2 by itself is completely unable to transduce a neuregulin signal in the absence of another ERB receptor with which to form heterodimer. This explained our previous data showing lack of a mitogenic response of the cultured NF-derived Schwann cells.

In summary, we extended our studies from the overexpression of one growth factor receptor, KIT, to a second growth factor receptor, PDGF. We found that this receptor was also overexpressed in both isoforms and that the cells could be stimulated to further proliferate when exogenous PDGF BB was added to the cells. This data raised the possibility that aberrant expression of growth factor receptors by Schwann cells such as the PDGF and cKit receptors, could represent an important step in the process leading to Schwann cell hyperplasia.

Preliminary Investigation of Intracellular Pathways Leading to Growth Factor Receptor Overexpression

As demonstrated in Figure 1, based on our current understanding of intracellular transduction pathways and the overexpression of both cKit and PDGF receptors, we have devised a rational flow sheet by which to explain the sustained proliferation which is characteristic of NF-derived Schwann cells. In particular, this pathway predicts that the key regulator, MAP kinase, will be hyperactivated in NF-derived Schwann cells since it will be activated by both the cKit and PDGF receptor pathway. We predict that this chronic hyperstimulation of MAP kinase activity leads to the phosphorylation of cytoplasmic phospholipase A2 a known substrate for MAP kinase. In turn, the phosphorylation and activation of phospholipase A2 releases arachidonate acid from

cellular phospholipids. The arachidonate acid can then be cyclized into both prostaglandins and thromboxanes which then can be released from the cell and interact with a prostaglandin type receptor. Prostaglandin receptor activation is closely linked with the activation of adenylate cyclase and the production of cAMP. Cyclic AMP in its own right is a mitogen for Schwann cells. In addition, Weinmaster and Lemke (35) have shown that chronic elevation of cAMP can lead to upregulation of PDGF receptors.

In summary, these pathways predict that the chronic stimulation of MAP kinase leads to elevated cAMP which will sustain the continued overexpression of receptors such as PDGF and cKit. The activation of these receptors in turn will continue to sustain the overactivation of MAP kinase. A circular metabolic pathway is set up which is synergistic and self-sustaining.

In order to fill in the gaps concerning downstream effects of activation of MAP kinase, we need to understand something about arachidonate acid metabolites and their effects on cultured Schwann cells. Thromboxane (TX A2) is a biologically active metabolite of arachidonate acid which can be produced by Schwann cells (36). The effects of TX A2 are mediated by a G protein coupled heptahelical receptor subfamily which have a molecular weight of 55 Kd. This receptor stimulates phospholipase C, resulting in inositol triphosphate accumulation (37). Although receptors for thromboxane have been identified in astrocytes (38) and oligodendrocytes (39), prior to our investigation, their existence in Schwann was completely unknown. Therefore, we analyzed both primary rat Schwann cells as well as the NF-derived Schwann cell line T265 to determine whether or not thromboxane receptors were present and if present, the metabolic effects of these receptors. The presence of the thromboxane receptor in the T265 cell line was confirmed in several ways. First, utilizing a series of thromboxane receptor specific antibodies raised in the laboratory of our collaborator, Dr. Guy Le Breton, we analyzed cell extracts from T265 by Western blotting. Our results our shown in the Figure 16 below.
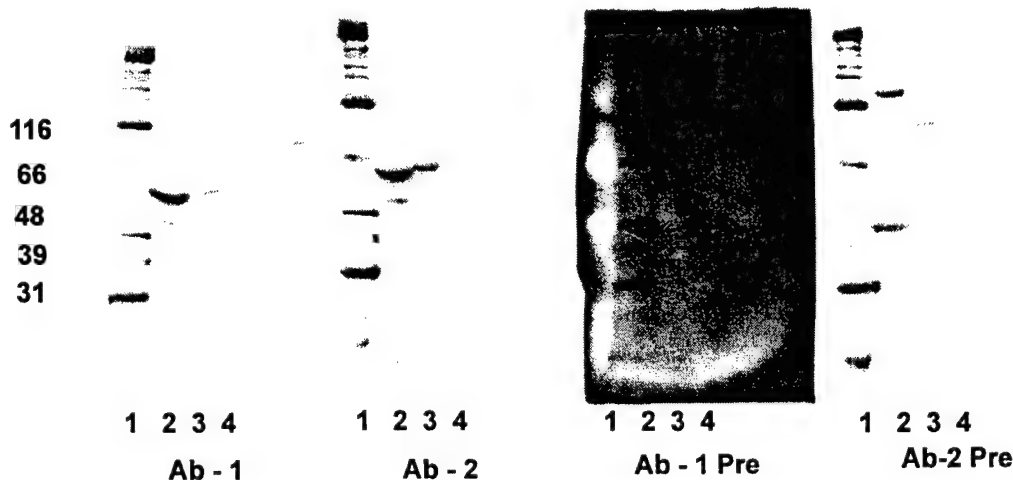

Fig16- Western blot analysis for TX receptor in cellular homogenates. Lane 1 = MW STDS, Lane 2 = platelets, Lane 3 = T265 cells, Lane 4 = CHO cells. Two antibodies to the Thromboxane receptor (Ab-1, Ab-2) were used along with corresponding preimmune serum controls (Ab-1 Pre, Ab-2 Pre).

Note that a strong immunoreactive band, having a molecular weight approximately equal to the well-characterized TX A2 receptor in the platelets was evident in the western blot analysis

of the T265 cell lines. In addition, we utilized these antibodies to immunostain the cells in culture. The cells demonstrated clear positive immunoreactivity, once again confirming the presence of the TX A2 receptor (data not shown).

What is the functional significance of this receptor? To test the functionality of the receptor, the following experiments were done. First, with respect to calcium metabolism, T265 cells were loaded with the methyl ester of the calcium sensitive indo dye followed by analysis on the Zeiss Attofluor Calcium Analysis System. The results are shown in Figure 17 below.
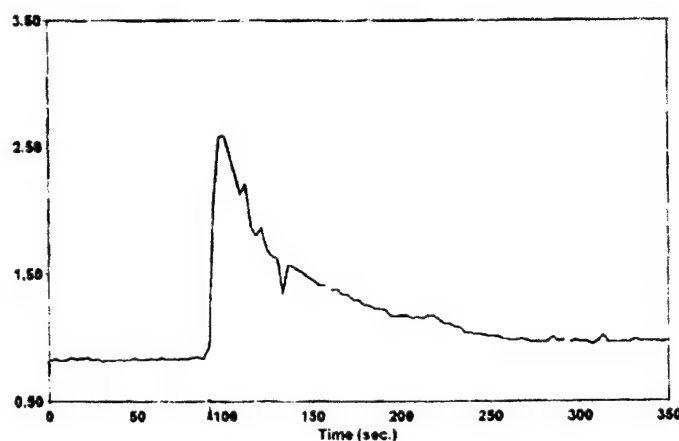

Figure 17- Calcium response of T265 cells to thromboxane A2 agonist. See text for experimental details.

After addition of the thromboxane A2 agonist, U46619, at a concentration of 5 μM, there was a very significant calcium response produced in the case of the T265 cells. On the other hand, under similar conditions, primary rat Schwann cells gave no response at all, although the cells were able to respond as shown by the positive ionomycin response carried out at the end of the experiment (data not shown). To further investigate the role of the thromboxane receptor and cAMP metabolism, the following experiment was carried out. U46619 was added at a concentration of 5 μM to either primary rat Schwann cells or the T265 cell line. Schwann cells were seeded onto 24 well culture plates at a density of 50,000 cells/well in DMEM with 10% FCS. Cells were serum deprived for 24 hours. Before treatment, Schwann cells were incubated with serum free media containing 1mM isobutyl methyl xanthine (IBMX) for 20 min to inhibit phosphodiesterase activity. After the media was aspirated, the cells were lysed with 0.1M HCl for 1 hour at room temperature on a rotary shaker. The lysate was collected in 500 ml centrifuge tubes and used immediately. Concentrations of cAMP were determined using the acetylated version of direct enzyme immunoassay kit (Assay Designs, Inc) according to the manufacturers instructions. The data was expressed as picomoles of cAMP per well. The concentrations of cAMP were determined in triplicate wells. The results are shown in Figure 18 below.
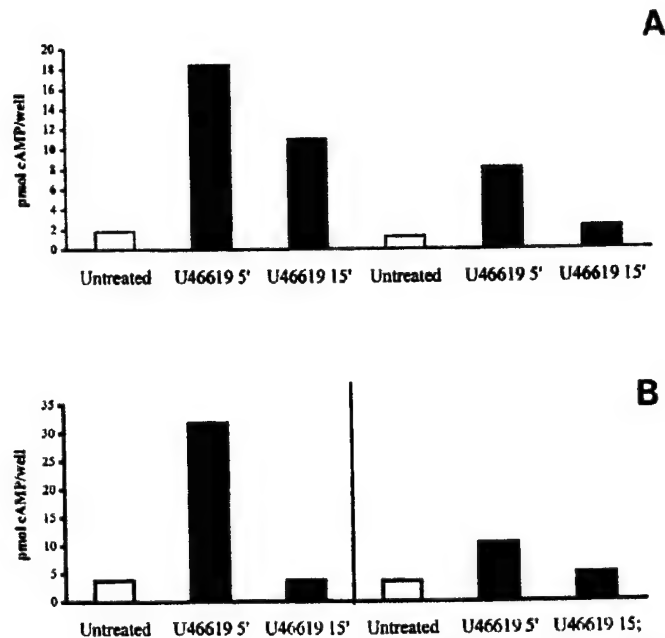
Figure 18- Effect of thromboxane agonist (U-46619) on intracellular cAMP levels in culutred Schwann cells. A. 5 uM U-46619 elicited a time-dependent increase in intracellular cAMP levels in both the T265 cell line (solid bars) and in primary rat Schwann cells (gray bars). B. Independent reptitions of the above experiment for each cell type. Treatments were performed in duplicate.

Note that there was a substantial increase in the level of cAMP in both the primary Schwann cells and the NF-derived Schwann cell line, T265, although the latter gave a more robust and sustained response. The next question was the extent to which activation of the thromboxane A2 receptor could stimulate cell proliferation. In this regard, the following experiment was carried out. T265 cells were seeded overnight in 96 well plates followed by treatment at various concentrations of the TXA2 agonist U46619. The proliferation of the cells was noted by utilizing the MTT assay after 48 treatments at each dose of the TXA2 agonist. The results are shown in Figure 19 below.
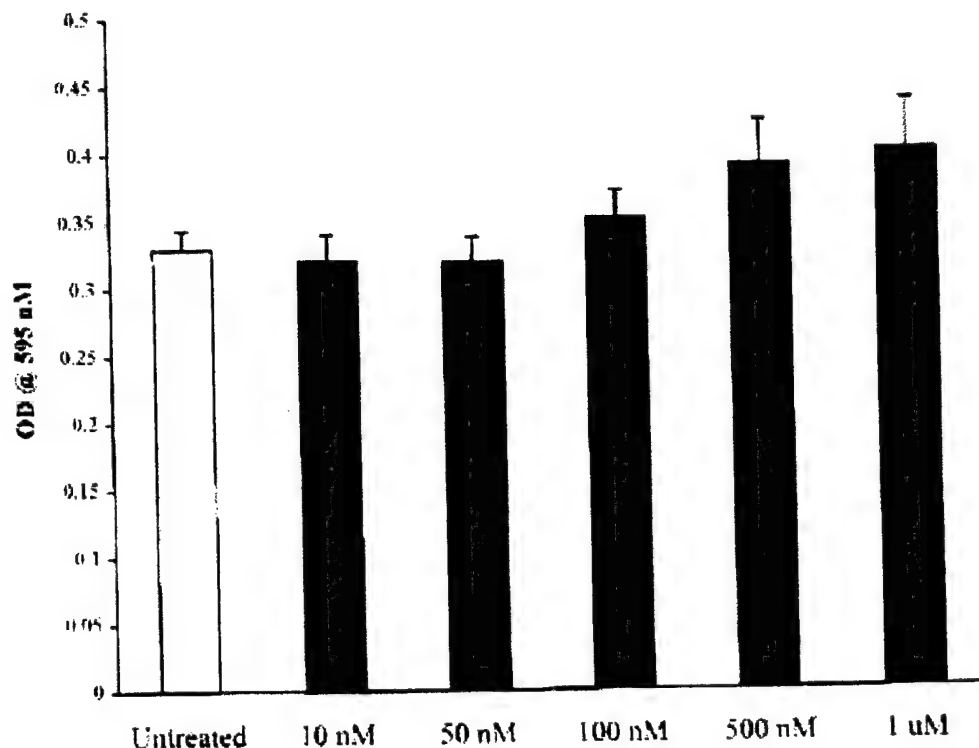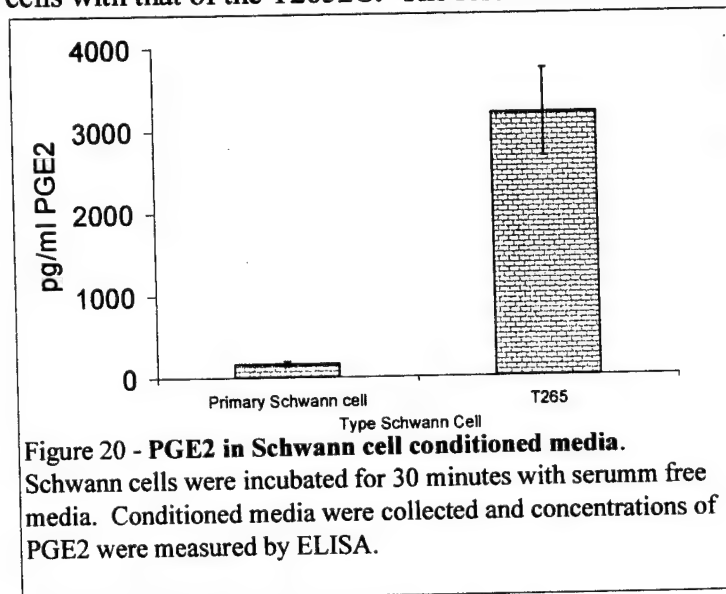
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Figure19 Dose dependent effect of U-46619 on the proliferation of the T265 cell line. Cells were seeded overnight onto 96 well plates at a density of 10,000 cells/well. The cells were serum deprived for 24 hours and then treated with U-46619 (n=10 for each dose) for 48 hrs. MTT (Boeringher Mannheim) was added for 4 hours prior to the termination of the experiment. The cells were lysed according to the manufacturers instructions and the optical density of each sample was measured at 595 nM.

Note that relative to the untreated cells, there was a dose dependent increase in cell number, particularly at the higher concentrations of agonist used. Therefore, it is reasonable to suppose that the presence of this receptor on the NF cell line is related in some way to the proliferative potential of the cells.

With respect to thromboxane receptors, our conclusions are as follows. The T265 cell line expresses a TXA2 receptor protein having a molecular weight consistent with that found in the well-characterized platelet TXA2 receptor. Stimulation of this receptor using the TXA2 agonist U46619 produced a 3-6 fold elevation of intracellular cAMP in primary rat Schwann cells and an 8-10 fold elevation of cAMP in the T265 cell lines within 5 minutes of addition. In the subsequent 10 minutes, cAMP levels decreased toward baseline. The TXA2 agonist, U46619 produced an increase in intracellular calcium levels only in the NF-derived Schwann cell line. There was a weak but significant effect on the proliferation of the T265 cells after treatment with the TXA2 agonist for 48 hours. These findings suggest that the pattern of coupling of the TXA2 receptor in the NF-derived Schwann is different from that of primary Schwann cells. It also implicates a potential role for elevation of intracellular calcium in the hyperplasia of Schwann
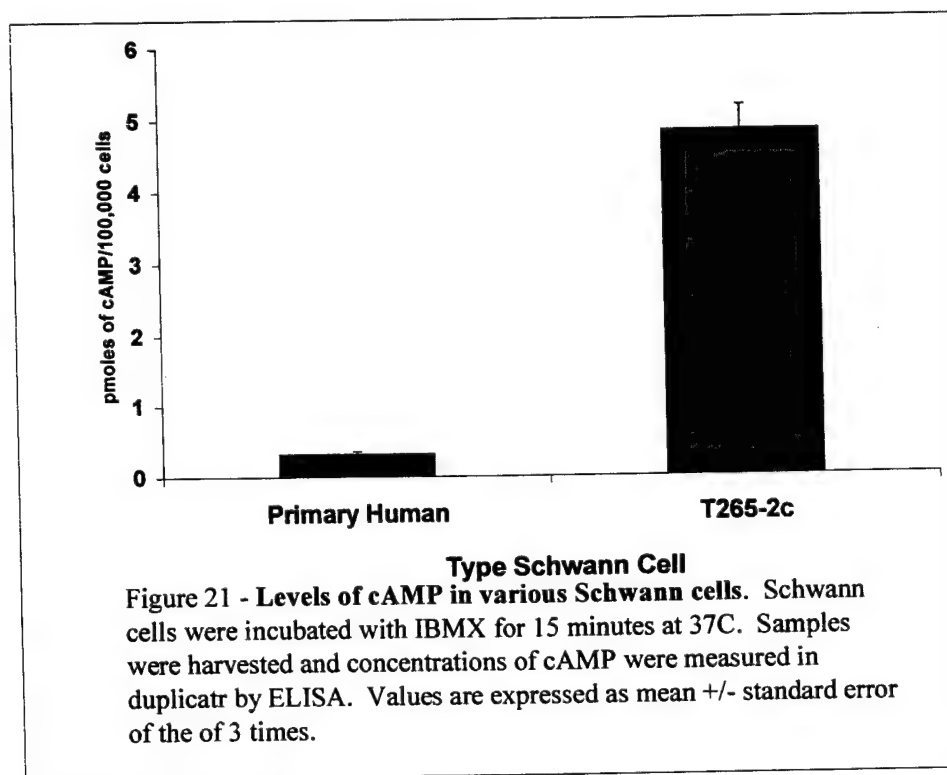
cells associated with NF-1. These findings fit well with our previous findings of the alteration in receptor metabolism specifically in NF-derived cell lines. In addition to overexpression of cKit and PDGFR, we now find that the prostaglandin receptor is uniquely coupled to elevation of calcium only in the NF-derived cell line. To summarize, at least one of the NF-derived Schwann cell lines contained a functional TXA2 receptor which was linked both to an increase in intracellular cAMP as well as an increase in intracellular calcium.

Next, we investigated the possibility that, according to our scheme shown in Figure 1, the NF cells were uniquely secreting arachidonate metabolites. In our initial experiment, T265 Schwann cells and primary Schwann cells were allowed to conditioned serum-free media for 24 hours. The media was then analyzed using a sensitive and specific ELISA assay kit (Assay Designs Inc.) for the presence of thromboxane. TXA2 was detected at a level of 1 nM in the T265 conditioned media but was completely absent in the conditioned media of primary rat Schwann cells. This data substantiated our hypothesis that excess arachidonate acid metabolism was occurring specifically in the T265 cells. Next, we analyzed Schwann cell media conditioned for 30 minutes in serum-free media for the presence of prostaglandins since these arachidonate metabolites are known to be involved in elevation of intracellular cAMP. Schwann cells were seeded onto 24 well culture plates at a density of 50,000 cells/well in DMEM with 10% FCS. Cells were deprived from serum for 24 hours. After 30 minutes, the supernatant was collected into a 96 well plates. Concentrations of PGE_2 were determined using the direct enzyme immunoassay kit (Assay Designs, Inc) according to the manufacturers instructions. The concentration of PGE_2 was determined in duplicate wells. We compared the conditioned media from primary Schwann cells with that of the T2652C. The results are shown in Figure 20 below.

Figure 20 - **PGE2 in Schwann cell conditioned media**. Schwann cells were incubated for 30 minutes with serumm free media. Conditioned media were collected and concentrations of PGE2 were measured by ELISA.

Note that there is a tremendous elevation of the prostaglandin PGE2 specifically in the T2652C cells relative to the conditioned media from primary Schwann cells. Note that although the level of TXA2 in conditioned media is not sufficient to significantly activate the receptor, but nevertheless, it is important to note its presence specifically in the transformed Schwann cells. However, the levels of PGE2 in conditioned media are fairly significant and high enough to activate the appropriate prostaglandin receptor.

Given the presence of TXA2 and PGE2, it was reasonable to ask whether or not cAMP was elevated in the T265 cell line. In primary human fetal Schwann cells and T265 Schwann cells were treated with the phosphodiesterase inhibitor, IBMX, for 15 minutes at 37°. The cells were then harvested and the concentration of cAMP in the cellular homogenate was measured in duplicate using the previously described sensitive ELISA analysis. The results are shown in Figure 21 below.

Figure 21 - **Levels of cAMP in various Schwann cells.** Schwann cells were incubated with IBMX for 15 minutes at 37C. Samples were harvested and concentrations of cAMP were measured in duplicatr by ELISA. Values are expressed as mean +/- standard error of the of 3 times.

Note that the levels of cAMP are elevated approximately 10 fold over that of primary human Schwann cells substantiating our hypothesis that elevated cAMP could in some way be responsible for some of the changes that we have noted in receptor expression in the cultured Schwann cells. In particular, chronic elevation of cAMP has been demonstrated to elevate PDGF receptor (35) and it is reasonable to suppose that a similar phenomenon occurs with the cKit receptor.

Thus, we now have experimental evidence for several aspects of us proposed pathway leading to proliferation in the NF-derived Schwann cells. Both thromboxane and prostaglandin A2 are secreted by NF-derived Schwann cells. The receptors for both of these arachidonate metabolites are also present and that intracellular cAMP is elevated presumably via activation of the prostaglandin and/or thromboxane receptor. Further experiments to clarify parts of this pathway are currently under way.

As noted in Figure 1, there are a number of intracellular transduction pathways which can be investigated to see how they are altered in the Schwann cells derived from neurofibromatosis. A particular powerful comparison can be obtained when looking at STS26T cells which contain neurofibromin and are dividing compared to the T265 and ST88 cell lines. We began our investigation by looking at the state of RAS activation in the various cell lines which lack

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neurofibromin. We were fortunate in having a former student, Dr. Simeng Suy, visit the lab and carry out a number of signal transduction assays with which she is very familiar having carried them out with respect to her work on the etiology of cancer (40,41,42). We first investigated the state of activation of RAS in the T265 cells, the ST88 cells, the STS26T cells, and human fetal Schwann cells. The results are shown in Figure 22 below.
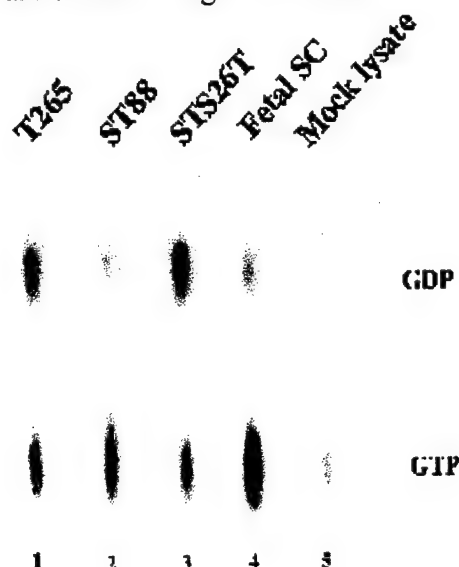

Figure 22 - Levels of Ras GTP in different Schwann cell types as labeled on the top of each lane.
Cells were lysed and whole cell extracts (1 mg) were immunoprecipitated with agarose-conjugated anti-H-Ras monoclonal antibody (259). To assess GTP binding and hydrolysis on Ras, the immunocomplexes were incubated with 1uCi of [α^{32}P]GTP (3000 Ci/mmol). The bound guanylnucleotides (GTP and GDP) were eluted and separated by thin layer chromatography followed by autoradiography. The migrations of GTP and GDP are as indicated.

T265 and ST88 have more activated RAS than the STS26T. Surprisingly, the human fetal Schwann cells have a high level of activated RAS perhaps due to the chronic stimulation with NDF-B which is required for their successful isolation. Therefore, we concluded that as has been reported for other cells, RAS is very activated in the NF-derived Schwann cell lines.

Another important transduction molecule is Raf, which is the next molecule downstream from activated RAS. We can evaluate the extend to which Raf was activated by immunoprecipitating Raf from T265 cells or STS26T cells followed by probing with an antibody to phosphorylated tyrosine since the consequence of RAS-Raf interaction is the production of a phosphorylated tyrosine in the Raf molecule. The results of that experiment are shown in Figure 23 below.

Figure 23

Raf phosphorylation in T265 cells (lane 1) and STS26T cells (lane 2).
Cells were lysed and whole cell lysates were immunoprecipitated with agarose conjugated anti Raf-1 antibody. Immunoprecipitated proteins were analyzed by immunoblotting using an anti-phosphotyrosine antibody.

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Note that in the STS26T, there is significant activation of Raf. Raf does not appear to be activated in the case in T265 cells even though there are high levels of RAS GTP. The significance of this is not clear at this point, but underscores the importance of investigating the alternate pathway for MAP kinase activation which involves phospholipase C and subsequent activation of MAP kinase by protein kinase C.

Next we investigated further the key intracellular component which is related to cellular proliferation, namely MAP kinase. Whole cell lysates (1mg of protein) were immunoprecipitated with agarose conjugated anti-ERK-2antibody (1ug) in 1 ml of lysis buffer overnight at 4°C with constant agitation. The ERK-2 immune complexes were washed three times with lysis buffer and assayed for MAP kinase activity according to the MAPK assay kit protocol (UBI). Briefly, the ERK-2 immunoprecipitates were washed once with assay dilution buffer (ADB: 20mM (MOPS) [pH 7.2], 25mM β-glycerol phosphate, 5 mM EGTA, 1mM Na_3VO_4, and 1mM DTT). The immune-complexes were resuspended in 10ul of ADB. The reactions were initiated by addition of 10 ul of the substrate cocktail (2 mg myelin basic protein (MBP) per ml of ADB), 10 ul of the inhibitor cocktail (20uM PKC inhibitor peptide, 2uM protein A inhibitor peptide (PKI), and 20uM compound, R24571), and 10ul of the magnesium-ATP cocktail (1 uCi [γ-^{32}P]ATP (3000 Ci/mmol) in 75mM magnesium chloride and 500 uM cold ATP). The reaction mixtures were then incubated for 15 min at 30°C with constant agitation. The reaction was stopped by blotting 10 ul of the reaction mixture, in triplicate onto P81 phosphocellulose paper. The filter papers were air dried and washed three times in a 50 ml conical tubes containing 40 ml of 0.75% phosphoric acid with gentle shaking on a rotator followed by an acetone wash. The filters were air dried and transferred to a scintillation vial containing 5 ml of scintillation fluid. The radioactivity that bound the filters was determined using a Beckman scintillation counter. In addition, 5ul of radiolabeled MBP was separated by electrophoresis and autoradiographed. The results are shown in Figure 24 below.
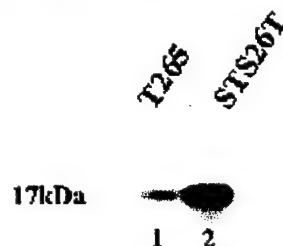

Figure24-MAPK kinase activity in T265 cells and STS26. Cells were lysed and whole cell extracts were immunoprecipitated with agarose conjugated anti ERK-2 antibody which recognizes both p42^{mapk} and p44^{mapk}. The MAPK immune complexes were incubated with 5 nmoles of [γ^{32}P]ATP and 20ug of MBP in 40 ul of the kinase reaction buffer. The radiolabelled MBP were separated by electrophoresis and autoradiographed.

Once again, we note that relative to T265 cell line, the STS26T cell line has a much higher level of intrinsic activation of MAP kinase indicating a difference in intracellular metabolism which is related to the presence or absence of neurofibromin. In order to evaluate the extent to which exogenous PDGF BB, shown to enhance the proliferation of the NF-derived

Schwann cells, could stimulate MAP kinase, the following experiment was carried out. The cells were serum-starved for 24 hours followed by stimulation with 20 mg/mol of PDGF BB for 5 minutes. The cells were lysed and the proteins were analyzed by immunoblotting with a phosphorylated MAP kinase antibody. The results are shown in Figure 25 below.
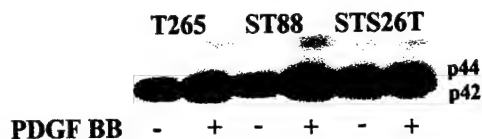

T265	ST88	STS26T
			p44
			p42

PDGF BB   -   +   -   +   -   +

Figure 25 - Phosphorylated MAPK in response to PDGF BB in T265, ST88, STS26T cell lines. Cells were starved and stimulated with 20ng/ml of PDGF for 20 minutes. Cells were lysed and proteins were analyzed by immunoblotting with phosphorylated MAPK antibody.

Note that in each case, the cells respond with an increased level of MAP kinase activity. However, the STS26T cells, which have a very active RAS pathway which stimulates MAP kinase activity, do not appear to respond the same as the ST88 or the T265 cells. These experiments taken collectively indicate important differences in intracellular signaling between cells lacking or containing neurofibromin and will form the basis of further investigations.

CONCLUSIONS

Based on our investigations to date, we have come to the following conclusions. We conclude that the metabolism of the transformed Schwann cells, derived from NF-1 patients, has been altered in the following ways:
1. Expression of growth factor receptors is greatly altered.
   A. c-Kit receptor is overexpressed.
   B. PDGF receptors are overexpressed.
   C. The receptors (erb B-3 receptor) required for NDF signal – transduction is absent
   D. Receptors for thromboxane are coupled to both increases in calcium metabolism as well as elevation of cAMP in contrast to normal Schwann cells in which Thromboxane receptors are coupled to increases in cAMP only.
2. Growth factor receptors which are overexpressed (c-Kit, PDGF-R) can be further activated by stimulation with the appropriate ligands (SCF, PDGF). This activation leads to increased proliferation.
3. No evidence of an autocrine pathway in NF-derived Schwann cells for the activation of these c-Kit by secreted SCF.
4. A viral transfection assay has been established in the laboratory, which in conjunction with the complete control inducible mammalian expression system available from Strategene, should enable us to immortalize human Schwann cells for further studies.
5. NF-derived Schwann cells lacking neurofibromin have increased arachidonate metabolism resulting in the secretion of TXA2 and PGE2.
6. The appropriate receptors for the secreted arachidonate metabolites are present in the Schwann cells derived from NF-1 patients.

7. There is a 10-fold basal elevation of intercellular cAMP in the NF-derived Schwann cells compared to normal human Schwann cells.

So What? (Knowledge as a Medical Product)

A major unanswered question in neurofibromatosis research is an understanding of changes in cellular metabolism which lead to the abnormal proliferation characteristic of neurofibromatosis. Our investigations have established for the first time that increased growth factor receptor expression is a consequence of the absence of neurofibromin in Schwann cells. We have also established, in a preliminary way, evidence for changes in intercellular transduction mechanisms including increased activity of phospholipase A2, secretion of arachidonate metabolites, and the elevation of intracellular cAMP in these cells. These metabolic changes help to perpetuate the hyperproliferative state characteristic of Schwann cells derived from neurofibromatosis patients. In turn, a better understanding of these abnormal pathways will lead to specific intervention techniques whereby the abnormal Schwann cell proliferation can be controlled.

References

1. V. M. Riccardi, *Arch. Dermatol.* **123**, 1011 (1987).
2. R. Giorno, J. Lieber, H. N. Claman, *Neurofibromatosis* **2**, 35 (1989).
3. J. Bienenstock *et al.*, *Am. Rev. Respir. Dis.* **143** (Suppl), S55 (1991).
4. P. Isaacson, *J. Pathol.* **119**, 193 (1976).
5. D. E. Williams *et al.*, *Cell* **63**, 167 (1990).
6. K. M. Zsebo *et al.*, *Cell* **63**, 213 (1990).
7. K. Jozaki *et al.*, *Exp. Hematol.* **19**, 185 (1991).
8. K. Nakajima *et al. Biochem. Biophys. Res. Commun.* **183**, 1076 (1992).
9. B. K. Wershil *et al. J. Exp. Med.* **175**, 245 (1992).
10. J. G. Flanagan, P. Leder, *Cell* **69**, 265 (1990).
11. E. Huang *et al.*, *Cell* **63**, 225 (1990).
12. D. E. Williams *et al.*, *Cell* **63**, 167 (1990).
13. E. Keshet *et al.*, *EMBO J.* **10**, 2425 (1991).
14. J. J. Ryan *et al.*, *J. Neurosci. Res.* **37**, 415 (1994).
15. G. I. Tennekoon *et al.*, *J. Cell Biol.* **105**, 2315 (1987).
16. D. A. Largaespada *et al.*, *Nature Genetics* **12**, 137 (1996).
17. B. Gideon *et al.*, *Nature Genetics* **12**, 144 (1996).
18. M. Sadao *et al.*, *J. Biol. Chem.* **270**, 28834 (1995).
19. T. Chang, J. D. McGrae, K. Hashimoto, *Pediatric Dermatol.* **10**, 224 (1993).
20. Y.K. Tay, *Dermatol.* **197**, 401 (1998).
21. S. J. Hines *et al.*, *Cell Growth and Differentiation* **6**, 769 (1995).
22. H. A. Kim, B. Ling, N. Ratner, *Mol. Cell. Biol.* **17**, 862 (1997).
23. R. Rago, J. Mitchen, G. Wilding, *Anal. Biochem.* **191**, 31 (1990).
24. M. Eilers *et al.*, *Nature* **340**, 66 (1989).
25. P. T. Krischmeier *et al.*, *DNA* **7**, 219 (1988).
26. Brown, A.M.C, and Scott, M.R.D. "Retroviral vectors." <u>DNA Cloning Vol. III. A Practical Approach</u>, Glover, D.M., ed. IRL Press, Washington. pp. 189-212.
27. A. S. Perkins, *Molec. Cell Biol.* **3**, 1123 (1983).
28. J. E. Yoshino *et al., J. Cell Biol.* **99**, 2309 (1984).
29. G. W. Krystal, S. J. Hines, C. P. Organ, *Cancer Res.* **56**, 370 (1996).
30. Y. Yamanishi et al., *Jpn J. Cancer Res.* **87**, 534 (1996).
31. C. Sato-Bigbee, G. H. DeVries, *J. Neurosci. Res.* **9**, 98 (1996).
32. E. L. R. Barry, F. A. Gesek, P. A. Friedman, *BioTechniques* **15**, 1018 (1993).
33. A. Badache, N. Muja, G. H. DeVries, *Oncogene* **17**, 795 (1998).
34. J. B. Davis, P. Stroobant, *J. Cell Biol.* **110**, 1353 (1990).
35. G Weinmaster, G. Lemke, *EMBO J.* **9**, 915 (1990).
36. A. L. Constable *et al.*, *Brain Res.* **635**, 75 (1994).
37. I Knezevic, C. Borg, G. C. Le Breton, *J. Biol. Chem.* **268**, 26011 (1993).
38. N. Nakahata *et al.*, *Brain Res.* **583**, 100 (1992).
39. S. C. Blackman *et al.*, *J. Biol. Chem.* **273**, 475 (1998).
40. U. Kasid *et al.*, *Nature* **382**, 813 (1996).
41. S. Suy *et al.*, *Oncogene* **15**, 53 (1997).
42. S. Suy *et al.*, *J. Biol. Chem.* **273**, 17871 (1998).
43. E. Hartree, *Anal. Biochem.* **48**, 422 (1972).

Experimental Neurology

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Cover photo. Human fetal Schwann cells align along neuritic processes of human fetal dorsal root ganglia neurons. See Lopez and DeVries, 1999, **158,** 1–8.

Isolation and Serum-Free Culture of Primary Schwann Cells from Human Fetal Peripheral Nerve

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We have developed a method for isolating Schwann cells (SC) from human fetal peripheral nerve and maintaining these SC *in vitro* under serum-free conditions. This method yields essentially pure SC which have a bipolar, spindle-shaped morphology; align in fascicles; and express typical glial cell markers. Human fetal SC can be maintained for months under serum-free conditions with the neuregulin NDFβ. These human fetal SC can mimic axonal contact *in vivo* by retaining the functional capacity to strongly associate with neurites of cultured human fetal dorsal root ganglia. These isolation, culture, and coculture techniques provide a method for investigating SC–neuron interactions as well as development and function of human fetal SC. © 1999 Academic Press

Key Words: human Schwann cells; dorsal root ganglia; *in vitro*; serum-free culture.

INTRODUCTION

Although Schwann cells (SC) function primarily as the myelinating cells of the peripheral nervous system, they have the ability to perform several other essential functions. SC can promote axonal regeneration (17, 23, 24), and SC reduce astrogliosis and cavitation (13) when transplanted into injured mammalian spinal cord. Besides their potential role in promoting spinal cord axonal regeneration (13, 22), SC have been found to participate in guiding sprouting axons to neuromuscular junctions following injury (20). In light of these observations, the ability to culture and maintain human SC *in vitro* will be essential to the use of SC in therapeutic transplantation strategies.

To date, the majority of *in vitro* studies of human SC have used pathology or autopsy specimens as a source of human adult SC (4, 10, 12–14, 17, 18, 23, 24). Such studies of human adult SC use mitogenic expansion to obtain pure SC (4, 12, 18) and maintain these SC in serum-containing medium (which permits fibroblast growth). Human fetal neural tissue has been the source of SC in a few studies (8, 9, 19), indicating that this

tissue can be a viable alternative to adult tissue. However, the methods reported for isolation and *in vitro* maintenance of human fetal SC are not sufficient to obtain pure populations of SC. We present a method by which to obtain essentially pure (>98%) human fetal SC and to maintain these SC *in vitro* for substantial lengths of time. We have studied the properties of normal fetal human SC which have been isolated and maintained *in vitro* under these defined conditions. We present an antigenic profile of these SC and also report experiments to test the capacity of these SC to associate with human fetal neurites in *in vitro* autologous cocultures. These methods provide a useful *in vitro* paradigm to purify SC and by which to investigate human SC–neuron interactions.

MATERIALS AND METHODS

Dissection of Peripheral Nerves

Tissue was obtained from normal concepti with no sign of neurological abnormalities at the Brain and Tissue Bank for Developmental Disorders at the University of Maryland School of Medicine (Baltimore, MD). Lower limb tissues containing peripheral nerves were stored at 4°C and shipped on ice for arrival within 36 h. Peripheral nerves were shipped *in situ*, and the sciatic nerve was dissected from within quadriceps and psoas muscles upon receipt. Nerves were washed three times in sterile RPMI (BRL) and blotted dry. Length and mass of nerves was determined and recorded. Mass was determined prior to nerve dissection because dissected fascicles are small and fragile to handle and manipulation of dissected fascicles risks significant tissue loss and introduces measurement error.

Nerves were cut into 1 to 2-mm lengths, and individual nerve fascicles were dissected free of connective tissues ensheathing individual nerve fascicles.

Isolation of Schwann Cells and Determination of Yield

Immediately, fascicles were dissociated in two digestion steps. Approximately 50 mg nerve tissue was

digested with an enzyme cocktail of freshly prepared dispase (25U, BRL) collagenase A (0.6–0.8 U, Serva) and hyaluronidase (1000 U, Sigma) in 2 ml DMEM (low glucose, BRL). Initial tissue digestion lasted 45–60 min at 37°C under constant agitation; another aliquot of collagenase A (0.6–0.8U) was added, and incubation then commenced for an additional 45–60 min.

Following digestion, defined N2 medium (3) supplemented with 10 ng/ml NDFβ (the β isoform of recombinant human neuregulin, Amgen) was added and the cells collected by centrifugation at <200g (1000 rpm in a Beckman Model T-J6 centrifuge) for 10 min. The supernatant was removed and cells were resuspended in NDFβ-supplemented N2. The number of living cells recovered was determined using a Neubauer hemocytometer.

Tissue Culture Conditions

To purify SC from contaminating fibroblasts, the dissociated cell suspension was placed into plastic culture dishes (Falcon) or culture dishes coated with Thy 1.1 antibody (ATCC) at 37°C. Cells settled for 2 hours (fibroblasts settling first) and the remaining cells in suspension (SC) were transferred to fresh culture dishes coated with laminin (Boehringer Mannheim). SC were maintained in N2 [Ref. 3; 50% DMEM with high glucose (BRL), 50% Ham's F-12 (BRL), 10μg/ml transferrin (human apo-transferrin, Sigma), 5 μg/ml insulin (Sigma), 1.4 mM L-glutamine (Sigma), progesterone (Sigma), putresceine (Sigma), sodium selenite (Sigma), and sodium bicarbonate (Sigma), 100 U/ml penicillin + 100 μg/ml streptomycin (Sigma), pH 7.2] supplemented with 10 ng/ml NDFβ (Amgen). Supplemented N2 culture medium (N2 + NDFβ) was changed every third or fourth day.

SC were trypsinized and replated to 12-mm-diameter thin glass coverslips as needed for immunocytochemistry. For immunochemical staining procedures, between 5000 and 20,000 SC were plated on laminin-coated, 12-mm-diameter thin glass coverslips (Fisher) in 24-well cluster plates (Costar). Wells containing SC on coverslips were flooded with 500 μl of supplemented N2 medium (3) 16 h after plating.

Dorsal Root Ganglion Isolation

Fetal human dorsal root ganglia (DRG) were obtained using the method of Wood (22). Briefly, DRGs were dissected from lumbosacral spinal cord, decapsulated, and dissociated for 45 min at 37°C in 2 ml Hanks' balanced salt solution containing 0.25% trypsin. Dissociated DRGs were collected by centrifugation and plated at a density of one DRG per five collagen-coated coverslips. Cultures were grown overnight in DRG culture medium (50/50 Ham's F12/DMEM, Gibco; 10% NuSerum, Collaborative Biosciences; 50 ng/ml NGF, 2.5S,

Boehringer Mannheim) and cycled three times with a 6-h exposure to the antimitotic fluorodeoxyuridine (140 μM) to eradicate native glia and fibroblasts. Under these conditions neurons thrive, neuritic processes grow, and no other cell types survive. Within 2 to 4 weeks a substantial number of neuritic processes having a length greater than 1 cm are present as a halo surrounding DRGs.

Schwann Cell and Neurite Coculture Conditions

After DRG neuritic processes grew 4 weeks, SC were seeded onto cultures (10,000–20,000 SC per DRG culture). DRG culture medium was supplemented to 10% final serum concentration with 50 μg/ml ascorbic acid for 2 days, and subsequent culture was continued in DRG culture medium + ascorbic acid as described (22) for the duration of coculture studies.

Preparation of Fixed Cells for Immunocytochemistry

Cells and cocultures were washed three times with Dulbecco's phosphate-buffered saline (DPBS, Gibco BRL) and fixed using a graded series of paraformaldehyde solutions (1, 2, 4% paraformaldehyde in DPBS). Cell preparations were again washed three times with DPBS and soaked in blocking solution (5% powdered milk/1% normal goat or bovine serum in DPBS) or permeabilizing block solution (5% powdered milk/1% normal goat or bovine serum/1% Triton X100 in DPBS) for 1 hour. Cover slips were then washed three times with DPBS to remove residual blocking solution.

Immunocytochemistry

Primary antibodies were diluted in DPBS (Table I) and cell preparations were exposed to primary antibody overnight. Cultures were washed three times in DPBS and incubated in blocking solution as before. Cultures were washed three times with DPBS and incubated with secondary antibody conjugates (lissamine rhodamine- or FITC-labeled conjugated IgG, Pierce) for 1 h. Cultures were washed three times with DPBS, dipped in sterile H$_2$O, and mounted cell side down in fluorescence-preserving mounting solution (n-propyl gallate, 5% in 1:9 PBS:glycerol) on glass microscope slides. Fluorescence microscopy was used to visualize positive staining of cells. Negative controls (no primary antibody) were included with every experiment, and data reported are the result of between three and six experiments. The purity of SC in culture was determined following immunocytochemistry by counting the number of SC labeled with the glial-specific S100 versus total number of cells in three fields. Following purification and immunochemical labeling, cultures were >98% SC ($n = 6$). In experiments with other markers, strength of immunofluorescent signal (values in Table II) was consistent in replicate experiments with human fetal SC.

RESULTS

Total Cell Yield and Recovery of Schwann Cells

Following dissection of nerves from appendicular muscle and connective tissues, mass was determined. Total nerve mass ranged from 0.03 to 0.15 g (Table I). Epineurium was dissected from nerve fascicles, fascicles were digested, and cell viability was determined

TABLE 1
Case Identification Code and Total Cell Yield

Sample ID no.	Gestational age (weeks)	Mass[a] (g)	Cells per gram tissue (in millions)
VA07a	18.5	0.08	17.5
VA07b	18.5	0.08	25
VA08a	20	0.05	18
VA08b	20	0.07	47
VA12a	19	0.06	67
VA12b	19	0.05	128.4
VA13a	19	0.04	132.5
VA13b	19	0.07	71.4
VA21	18	>0.02	1.46[b]
VA22a	17	>0.02	2.3[b,c]
VA22b	17	>0.02	2.3[b,c]
VA23a	19	0.03	165
VA23b	19	0.05	72
VA24a	18	0.02	155
VA24b	18	0.05	70
VA25a	N.A.	0.05	72
VA25b	N.A.	0.06	45
VA26a	21	0.06	46.7
VA26b	21	0.06	74.2
VA27a	17	0.06	68.3
VA27b	17	0.03	43.3
VA28a	22.5	0.06	56
VA28b	22.5	0.10	60
VA29a	19	0.04	215
VA29b	19	0.04	95
VA30	19	0.03	75
VA31	17	0.04	34
VA32a	23	0.08	81.3
VA32b	23	0.15	85.3
VA34a	19.5	0.03	39
VA34b	19.5	0.02	88.5
VA38	19	0.10	105
VA41	18	0.10	111
VA42	19.5	0.11	115.5
VA44	19.5	0.08	175
VA45	16.5	0.04	238
VA47	18	0.09	102
VA48	19.5	0.08	107
VA49a	18.5	0.06	200
VA49b	18.5	0.04	137.5
VA50	19	0.07	166
VA54	19	0.13	111.5
VA55	17	0.03	190
VA56	18	0.04	230

Note. N.A., gestational age data not available.
[a] Mass recorded before removal of connective tissue.
[b] Total number of cells recovered rather than cells per gram tissue.
[c] Nerve samples were combined prior to digestion.

TABLE 2
Antigenic Profiles of Fetal Human Schwann Cells

Antibody raised to: Antigen ([a], [b])	Relative reactivity[c]
S100 (P, Dako)	+++
P0 (M, C. Linington)	++
MBP (P, G. Deibler)	++
O1 (M, hybridoma supernatant, S. Pfeiffer)	++++
O4 (M, hybridoma supernatant, S. Pfeiffer)	++
GFAP (M, Sigma)	++
CNPase (M, ascites fluid, T. Sprinkle)	+
Laminin (M, Sigma)	−
p75 (M, hybridoma supernatant, ATCC)	+++
Neurofilament (M, Sternberger)	−
Human fibroblast surface protein (M, Sigma)	−

[a] P, polyclonal antibody, M, monoclonal antibody.
[b] Source of antibody.
[c] No immunoreactivity (−) and slightly immunoreactive (+) to highly immunoreactive (++++).

by trypan exclusion assay immediately after fascicles were completely digested. The total cell yield from nerve samples was 94×10^6 cells per gram of tissue ($\pm 61 \times 10^6$ SD, <5% dead cells in each experiment, $n = 44$).

Variation in cell recovery is due to a number of factors, including variability in postmortem time interval (17–36 h), gestational age of tissue, as well as the lowest SC yields from extremely small nerve samples (Table I). Additionally, cell recovery increased dramatically as expertise was gained developing the technique, with lower cell yields from initial trials and consistently higher cell yields as dissection skills increased and dissociation techniques were optimized.

Prior to panning and serum-free culture, approximately 80% of recovered cells are SC as determined by immunocytochemistry using an antibody to the SC protein S100 (vide infra). Schwann cells were separated from contaminating fibroblasts by differential adhesion and by maintenance in serum-free N2 medium (3) supplemented with the growth factor NDFβ (10 ng/ml; see Materials and Methods). Schwann cells maintained under these defined, serum-free conditions appear morphologically normal, and survive for weeks in vitro with culture medium changed every third or fourth day (Fig. 1).

Identification and Immunocytochemical Analyses of Schwann Cells

The antigenic profile of human fetal SC in vitro was determined via immunocytochemistry. We used the glial-specific marker, S100, as a positive marker for fetal human SC in primary populations of cells isolated from nerve (Fig. 2b). Following purification, the proportion of S100-positive cells (SC) in cultures is consistently 98–99% (Figs. 2b and 3). In addition to express-

FIG. 1. Human fetal Schwann cells maintained *in vitro* for 8 weeks. After long periods of *in vitro* culture, the SC orient in palisades and align in fascicles, visibly swirling across the culture dish. SC were maintained under serum-free conditions in N2 medium (3) supplemented with 10 ng/ml NDFβ$_1$. Bar, 15 μm.

ing the calcium-binding protein S100 (Fig. 2b), human fetal SC also express the glial marker protein galactocerebroside (O1-positive, Fig. 2d), the myelin constituent proteins P0 and myelin basic protein, the intermediate filament glial fibrillary acidic protein (GFAP), CNPase, and the low-affinity nerve growth factor receptor p75 and are immunoreactive with the O4 antibody (an antibody which recognizes sulfatide, cholesterol, and seminolipid; Fig 2f, Table II). Fibroblasts were negative for S100 staining (Fig. 2b). In addition to negative controls with each immunolabeling experiment, cultures were probed with an antibody to human fibroblast surface protein (Sigma), and while the few remaining fibroblasts were labeled, there was no labeling of SC in primary cultures (Table II).

The antigenic profile of fetal human SC is similar to that of human adult SC in that adult SC also are immunoreactive to S100, P0, myelin basic protein, and O4. In contrast, the early glial marker GFAP is barely detectable in adult human SC *in vitro* (19) but is present on fetal human SC. Another difference is that adult SC express laminin; laminin could not be detected by immunocytochemistry of human fetal SC maintained in serum-free conditions, although human

fetal SC maintained in the presence of fetal bovine serum do express laminin (9).

Human Fetal Schwann Cells Associate with Human Fetal DRG Neurites in Coculture

The above observations that fetal human SC express some myelin component proteins suggest that fetal SC might be myelin competent. To myelinate axons, SC must first associate with these neuronal processes. To test whether fetal human SC would associate with neurites *in vitro,* cultures of human fetal DRG neurons were established, showing extensive neuritic outgrowth. Upon seeding SC onto neuron cultures, SC migrated and preferentially attached to neurites (Fig. 3). Within days all SC had migrated to neurites and were tightly in contact with neuritic processes (Fig. 3). After 4 weeks in coculture, we performed immunocytochemical analyses, staining SC for S100 and neurites for neurofilament protein (Fig. 2). Double immunolabeling of SC + neurite cocultures (Fig. 3) showed that neurofilament labeling was limited to neuron cell bodies and neurites (fluorescein-labeled, Fig. 3) and SC (anti-S100, rhodamine) affixed to neurite projections.
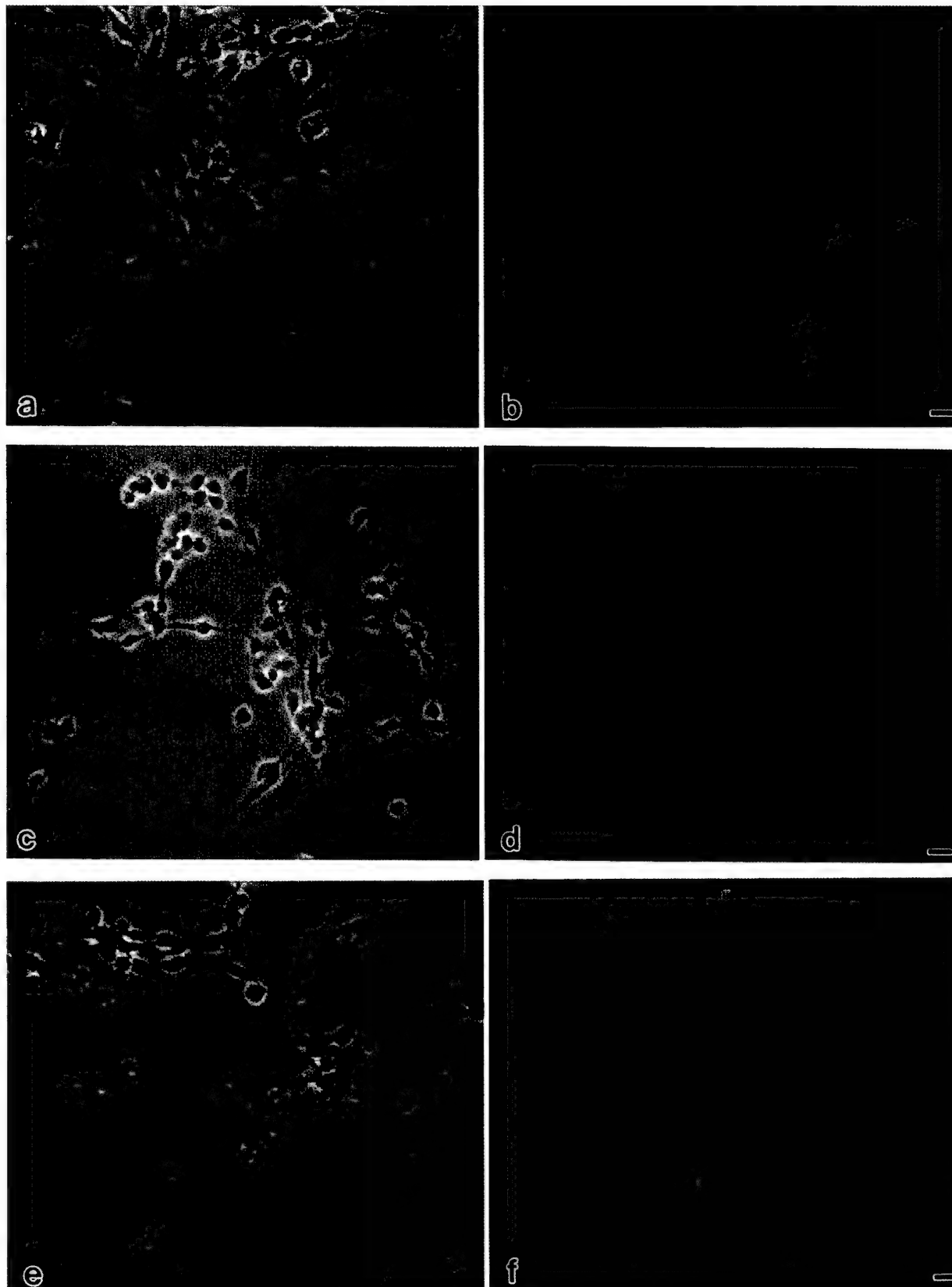
FIG. 2. Human fetal Schwann cells (SC) express typical SC antigens. Bars, 15 μm. (a, c, e) Phase contrast micrographs of SC after 2 days *in vitro*. The SC are immunoreactive to primary antibodies to (b) S100, (d) O1, and (f) O4. (b, d, f) Fluorescent images of SC labeled with either lissamine–rhodamine- (b) or fluorescein isothiocyanate- (d, f) conjugated secondary IgG.
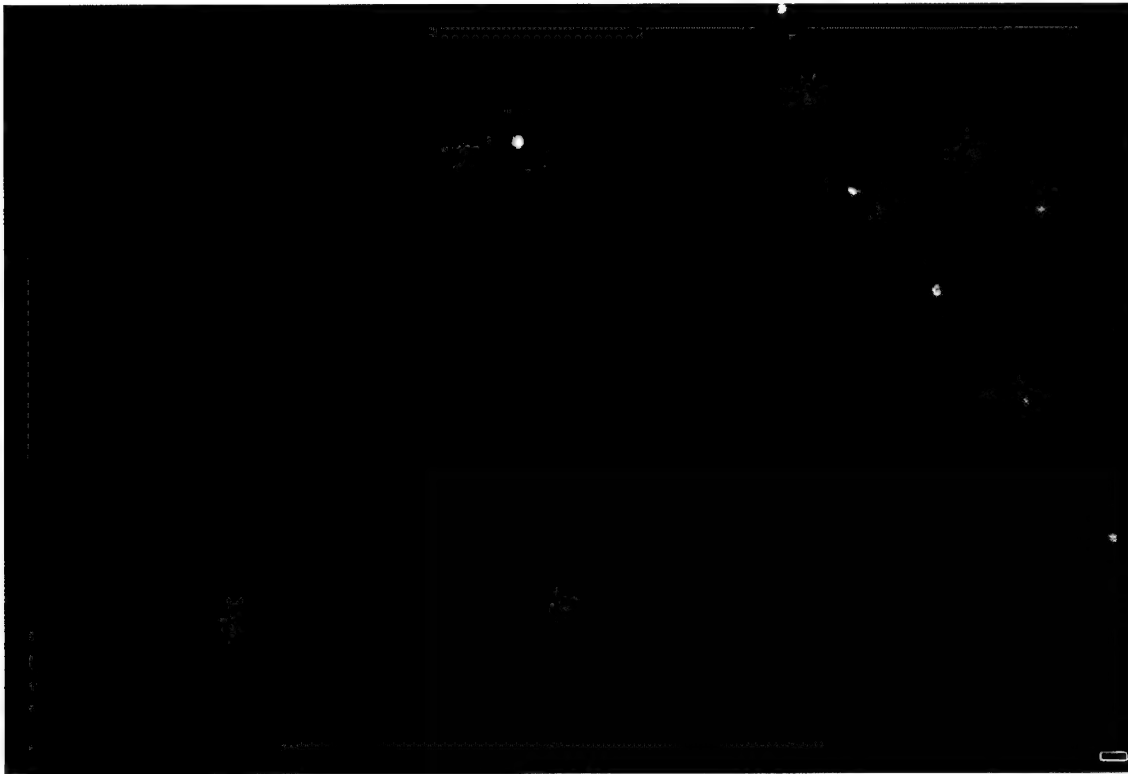
FIG. 3. Human fetal Schwann cells associate with neurites *in vitro*. Bar, 5 μm. Fetal human SC were isolated and grown *in vitro* for 30 days and seeded onto DRG neurite cultures. The accompanying double-exposure photomicrograph demonstrates that in homologous coculture of human fetal SC with human fetal DRG neurons, SC preferentially associate with neurites. Differential immunocytochemical staining of SC and DRG neurites in coculture in the image has been achieved by labeling SC with a rabbit polyclonal antibody to S100 and a rhodamine-conjugated secondary anti-rabbit IgG and labeling neurites with a monoclonal antibody to the low-affinity nerve growth factor receptor and a fluorescein-conjugated anti-mouse IgG secondary antibody. Where parallel rows of SC appear out of the plane of focus, SC appear as strings of red (S100-positive) cells; however, the immunofluorescent signal of the neurofilament-positive neurites to which these SC attach cannot be seen as readily as the neurites in the center of this field.

Rows of parallel SC not in the precise plane of focus can also be discerned in Fig. 3. Due to the small caliber of the neurites to which these SC relate, fluorescence signal of neurofilament on these neurites can be discerned only when neurites are in the plane of focus (Fig. 3).

DISCUSSION

To be useful in transplantation therapies, sufficient numbers of pure SC must be readily available. Using this method, millions of SC can be obtained from a single nerve preparation (Table I). The differences in cell yield are due to several factors. The number of SC decreases from proximal to distal portions of sciatic nerve. Gestational age of nerve tissue also affects the number of SC present, a factor that could not be kept constant. Age also affects whether SC have undergone the wave of proliferation associated with axonal contact.

The cell population recovered is heterogeneous (SC + fibroblasts), SC comprising the majority of cells. We find the proportion of SC in initial cells recovered from nerve before purification to be <80% by immunocytochemical analyses and raw cell counts. The proportion of SC in the heterogeneous cell mixture from adult sciatic and sural nerve biopsies is greater, approaching 90% (16, 18). However, studies using human adult tissue (12, 15, 16, 18) employ strategies involving explanting nerves (where fibroblasts migrate out of nerve prior to SC isolation) and/or mitotic pressure to achieve highly pure (>95% pure) adult human SC cultures. The current protocol avoids maintaining nerve explants for extended periods of time in culture to obtain very pure cultures of adult SC (>95%, Refs. 11, 18) and to eliminate fibroblasts. The most profound difference between this technique and other techniques is that this procedure is done under defined conditions and avoids the use of serum. Also, this technique avoids the temporal delay associated with protocols for isolating and obtaining pure SC from adult tissue (9, 11), a consideration which may be suboptimal for obtaining SC for transplantation.

In the procedure we have devised, purified SC (Fig. 1) are generated in successive steps. Early removal of

fibroblasts is accomplished by allowing fibroblasts to settle from the suspension for 2 h, and the SC in suspension are removed to a fresh culture dish. This method alone is effective in producing relatively pure (above 90%, data not shown) cultures of SC.

Growth substrata affect fetal SC yield and survival. Our initial attempts to isolate human fetal SC used different substrata for SC growth (fibronectin, laminin, collagen, poly-D-lysine, and poly-L-lysine). We found laminin to be the best substratum to promote human fetal SC adherence and increased SC recovery (data not shown). We also found that SC can be grown on collagen; however these SC remain only weakly affixed after 3 to 6 days. SC do not survive under our defined conditions on polylysine-coated dishes, and fibroblasts survive in cultures plated on fibronectin, probably due to the presence of serum-derived factors.

The elimination of contaminating fibroblasts also involved maintenance of fetal human SC in defined N2 medium (3) supplemented with NDFβ (10 ng/ml). Serum-containing medium permits fibroblast proliferation, where fibroblasts shortly outnumber the SC. Selective culture of SC in N2 medium supplemented with NDFβ works well for several reasons. Supplemented N2 does not permit fibroblast proliferation or survival. While fetal rat glia can survive in vitro (3) in serum-free N2 alone, under these conditions human fetal SC do not survive. For optimal survival of human fetal SC, we find that it is essential to supplement N2 medium with NDFβ. Initial attempts at human fetal SC isolation and culture showed NDFβ-supplemented N2 medium (3) to be better than other serum-free culture media tested (DMEM, DME, or MEM, data not shown). We also find that of the neuregulin isoforms tested (α_1, α_2, β_1, β_2, β_3) the β-neuregulin isoform, NDFβ$_1$, permitted optimal SC survival (data not shown). Populations of SC which are >98% pure can be continuously maintained in N2 + 10 ng/ml NDFβ for considerable periods of time (Fig. 1, SC at 8 weeks in vitro).

The involvement of the neuregulins (in our case, the growth factor NDFβ$_1$) in the growth and development of glia is expected; NDFβ has been found to promote fetal SC survival in vivo (2) and to play a role in neuronal survival and neurite outgrowth in vitro (21). However, we did not expect NDFβ to be essential for continuous serum-free culture of human fetal SC (unnecessary for rodent glia in vitro). Our laboratory and other studies have shown the β isoform of neuregulin to be a mitogen for adult human SC (11, 12, 15). NDFβ provides a twofold "bias" (namely, by having both proliferative effects (12) and survival effects reported herein) in promoting growth of highly pure SC cultures.

It is significant that integral proteins of peripheral nervous system myelin (myelin basic protein and P0) are expressed by fetal SC. However, unlike other mammalian fetal SC, laminin is absent in human fetal

SC (Table II) cultured in this manner. Several explanations for this are plausible. The SC used may be at an immature stage, at a time during which SC do not produce detectable levels of laminin; the serum-free culture conditions outlined here may not contain necessary components or signals for production of some proteins (rat fetal SC require axonal contact plus ascorbic acid to produce laminin; Ref. 7); also, human fetal SC maintained in the presence of serum express laminin (9). Isolated SC used in this study were from sciatic nerve and not in contact with DRG neurites as in (9); thus these SC may be undergoing a process of dedifferentiation resultant of culture.

In summary, we have developed a method for isolation and retrieval of essentially pure SC from fetal tissues. The growth conditions and substrata for isolating fetal human SC are markedly different from other published techniques used for adult human SC. While these SC express many of the signature protein markers of mature SC, they lack the mature phenotype of adult SC (GFAP is present; laminin is absent). This observation might be attributable to the early developmental stage of the source tissue (gestational age 17–23 weeks) or to the stringent in vitro culture regime. We have also shown that these SC strongly associate with human neurites in vitro, a functional prelude to remyelination that must be achieved if SC are to be used in successful transplantation therapies. We have been able to maintain these fetal human SC under in vitro culture conditions for long periods of time, a strategy which may be advantageous in developing a consistently available reserve of tissue for transplantation. While SC must meet several other functional criteria (especially for remyelination and restoration of function), our studies indicate that human fetal SC are an obvious choice for further functional assays to assess their potential for therapeutic transplantation. This method of human fetal SC isolation and the preliminary studies demonstrating strong association with neurites in vitro indicate that purified fetal human SC might hold promise for transplantation therapies.

The next obvious step is to use these SC–neuron cocultures under classical conditions for myelination to occur, to further test whether these SC are myelin-competent. Studies are under way to develop and optimize in vitro myelination conditions for human neurons and SC and to examine the functional capacities of SC, isolated in this way, to myelinate human neurons.

REFERENCES

1. Archer, D. R., P. A. Cuddon, D. Lipsitz, and I. D. Duncan. 1997. Myelination of the canine central nervous system by glial cell transplantation: A model for repair of human myelin disease. *Nature Med.* **3:** 54–59.

2. Bermingham-McDonogh, O., K. L. McCabe, and T. A. Reh. 1996. Effects of GGF/neuregulins on neuronal survival and neurite outgrowth correlate with erbB2/neu expression in developing rat retina. *Development* **122:** 1427–1438.

3. Bottenstein, J. E., and G. H. Sato. 1979. Growth of a rat neuroblastoma cell line in serum-free supplemented medium. *Proc. Natl. Acad. Sci. USA* **76:** 514–517.

4. Casella, G. T. B., R. P. Bunge, and P. M. Wood. 1996. Improved method for harvesting human Schwann cells from mature peripheral nerve and expansion *in vitro*. *Glia* **17:** 327–338.

5. Cheng, H., Y. Cao, and L. Olson. 1996. Spinal cord repair in adult paraplegic rats: Partial restoration of hind limb function. *Science* **273:** 510–513.

6. Duncan, I. D. 1996. Glial cell transplantation and remyelination of the central nervous system. *Neuropathol. Appl. Neurobiol.* **22:** 87–100.

7. Eldridge, C. F., M. B. Bunge, R. P. Bunge, and P. M. Wood. 1987. Differentiation of axon-related Schwann cells *in vitro*. I. Ascorbic acid regulates basal lamina assembly and myelin formation. *J. Cell Biol.* **105:** 1023–1034.

8. Kim, S. U., G. Moretto, V. Lee, and R. K. Yu. 1986. Neuroimmunology of gangliosides in human neurons and glial cells in culture. *J. Neurosci. Res.* **15:** 303–321.

9. Kim, S. U., V. W. Yong, K. Watabe, and D. H. Shin. 1989. Human fetal Schwann cells in culture: Phenotypic expressions and proliferative capability. *J. Neurosci. Res.* **22:** 50–59.

10. Levi, A. D. O. 1996. Characterization of the technique involved in isolating Schwann cells from adult human peripheral nerve. *J. Neurosci. Methods* **68:** 21–26.

11. Levi, A. D. O., R. P. Bunge, J. A. Lofgren, L. Meima, F. Hefti, F. Nikolics, and M. X. Sliwkowski. 1995. The influence of heregulins on human Schwann cell proliferation. *J. Neurosci.* **15:** 1329–1340.

12. Li, R.-H., J. Chen, G. Hammonds, H. Phillips, M. Armanini, P. Wood, R. Bunge, P. J. Godowski, M. X. Sliwkowski, and J. P. Mather. 1996. Identification of Gas6 as a growth factor for human Schwann cells. *J. Neurosci.* **16:** 2012–2019.

13. Martin, D., P. Robe, R. Franzen, P. Delrée, J. Schoenen, A. Stevenaert, and G. Moonen. 1996. Effects of Schwann cell transplantation in a contusion model of rat spinal cord injury. *J. Neurosci. Res.* **45:** 588–597.

14. Moretto, G., S. U. Kim, D. H. Shin, D. E. Pleasure, and N. Rizzuro. 1984. Long-term cultures of human adult Schwann cells isolated from autopsy materials. *Acta Neuropathol.* **64:** 15–21.

15. Morrissey, T. K., R. P. Bunge, and N. Kleitman. 1995. Human Schwann cells *in vitro*. II. Myelination of sensory axons following extensive purification and heregulin-induced expansion. *J. Neurobiol.* **28:** 190–210.

16. Morrissey, T. K., N. Kleitman, and R. P. Bunge. 1995. Human Schwann cells *in vitro*. I. Failure to differentiate and support neuronal health under co-culture conditions that promote full function of rodent cells. *J. Neurobiol.* **28:** 171–189.

17. Morrissey, T. K., A. D. O. Levi, A. Nuijens, M. X. Sliwkowski, and R. P. Bunge. 1995. Axon-induced mitogenesis of human Schwann cells involves heregulin and p185^{erbB2}. *Proc. Natl. Acad. Sci. USA* **92:** 1431–1435.

18. Rutkowski, J. L., G. I. Tennekoon, and J. E. McGillicuddy. 1992. Selective culture of mitotically active human Schwann cells from adult sural nerves. *Ann. Neurol.* **31:** 580–586.

19. Scarpini, E., B. Q. Kreider, R. P. Lisak, G. Meola, M. E. Velicogna, P. Baron, S. Beretta, M. Buscaglia, A. H. Ross, and G. Scarlato. 1988. Cultures of human Schwann cells isolated from fetal nerves. *Brain Res.* **440:** 261–266.

20. Son, Y.-J., J. T. Trachtenberg, and W. J. Thompson. 1996. Schwann cells induce and guide sprouting and reinnervation of neuromuscular junctions. *TINS* **19:** 280–285.

21. Trachtenberg, J. T., and W. J. Thompson. 1996. Schwann cell apoptosis at developing neuromuscular junctions is regulated by glial growth factor. *Nature* **379:** 174–177.

22. Wood, P. M. 1976. Separation of functional Schwann cells and neurons from normal peripheral nerve tissue. *Brain Res.* **115:** 361–375.

23. Xu, X. M., A. Chen, V. Guénard, N. Kleitman, and M. B. Bunge. 1997. Bridging Schwann cells transplants promote axonal regeneration from both the rostral and caudal stumps of transected adult rat spinal cord. *J. Neurocytol.* **26:** 1–16.

24. Xu, X. M., V. Guénard, N. Kleitman, and M. B. Bunge. 1995. Axonal regeneration into Schwann cell-seeded guidance channels grafted into transected adult rat spinal cord. *J. Comp. Neurol.* **351:** 145–160.

SHORT REPORT

Expression of Kit in neurofibromin-deficient human Schwann cells: role in Schwann cell hyperplasia associated with Type 1 Neurofibromatosis

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Type 1 Neurofibromatosis (NF1) is characterized by the formation of neurofibromas, benign tumors composed mainly of Schwann cells, which can turn malignant to form neurofibrosarcomas. Neurofibromin, the protein product of the *Nf1* gene, is believed to act as a tumor suppressor, accelerating the conversion of the oncogene Ras to its inactive form. The absence of neurofibromin could therefore lead to higher Ras activity in Schwann cells, resulting in uncontrolled growth through a cascade of events not yet elucidated. We describe the abnormal expression of high levels of the Kit tyrosine kinase receptor in both NF1-derived Schwann cell lines and tissue, as compared to primary Schwann cells or schwannoma-derived cells. High levels of Kit expression in the neurofibrosarcoma-derived Schwann cells correlate with a decrease in neurofibromin expression. Using inhibitors of tyrosine kinase receptors, we found that proliferation of the neurofibrosarcoma-derived cells is dependent upon activation of a subclass of tyrosine-kinase receptors. The proliferation of these cells is not dependent upon an autocrine loop involving typical Schwann cell mitogens. Finally, the proliferation of the neurofibrosarcoma-derived Schwann cells can be increased by stimulation with Kit ligand. These data implicate Kit as one of the components leading to the Schwann cell hyperplasia observed in NF1.

Keywords: neurofibromatosis; Schwann cells; c-*kit* proto-oncogene; stem cell factor; growth factors

Neurofibromatosis Type 1 (NF1) is a human genetic disorder affecting approximately one in 3000 individuals that manifests with various phenotypic features, including neurofibromas, café-au-lait spots, axillary freckling, Lisch nodules, optical gliomas, skeletal abnormalities and learning disabilities (Riccardi, 1991). Neurofibromas are benign peripheral nerve sheath tumors, which are comprised primarily of Schwann cells, but also contain fibroblasts, perineurial cells and mast cells embedded in an abundant extracellular matrix. Patients with NF1 are at risk for development of malignant Schwann cell tumors, called neurofibrosarcomas, which often arise from a preexisting neurofibroma (Ducatman et al., 1986; Halling et al., 1996). The *Nf1* gene, mutated in NF1, was

identified by positional cloning (Cawthon et al., 1990; Viskochil et al., 1990; Wallace et al., 1990) and its protein product, neurofibromin, was found to be expressed at highest abundance in neurons, Schwann cells and oligodendrocytes (Datson et al., 1992). Because the *Nf1* gene shares sequence homology with genes coding for Ras GTPase activating proteins (Buchberg et al., 1990; Xu et al., 1990), it was hypothesized that in the absence of functional neurofibromin, Ras deregulation could lead to tumor formation in NF1 (Basu et al., 1992; DeClue et al., 1992). However, introduction of activated Ras into normal Schwann cells resulted in growth arrest (Ridley et al., 1988) and Schwann cells from neurofibromin deficient mice exhibit activation of Ras and inhibition of cell proliferation (Kim et al., 1995). Therefore mutation in NF1 itself cannot explain NF1-Schwann cell hyperplasia and other factors underlying the development of NFS still need to be investigated.

Stem cell factor (SCF) is a growth factor required for the development of hematopoietic stem cells, primordial germ cells and melanocytes (Williams et al., 1992). We have previously shown that normal Schwann cells, as well as a neurofibrosarcoma (NFS)-derived cell line express SCF (Ryan et al., 1994). The receptor for SCF is the protein product of the proto-oncogene *kit*, which belongs to the platelet-derived growth factor (PDGF) subgroup of tyrosine kinase receptors (Yarden et al., 1987). Interestingly, Kit was not found in normal Schwann cells, but was expressed in the NFS-derived Schwann cell line, raising the possibility of an autocrine stimulated growth (Ryan et al., 1994). Therefore, it was of interest to further investigate the role of Kit in the development of Schwann cell tumors associated with NF1. In this study, we analysed both Kit and neurofibromin expression in primary Schwann cells, in Schwann cell lines derived from NF1-associated tumors and in a Schwann cell line derived from a sporadic schwannoma. We also investigated the possible contribution of tyrosine kinase receptor activation, in general, and Kit activation, in particular, to the hyperproliferative process leading to Schwann cell hyperplasia in NF1.

In order to show that expression of high levels of Kit is a feature specific for Schwann cells derived from NF1 patients, we evaluated the expression of Kit in four different Schwann cell lines, by Western blotting. Three of the cell lines, ST88-14, T265-2c and NF1T were derived from neurofibrosarcomas resected from patients diagnosed with NF1. The fourth cell line, called STS-26T, was isolated from an isolated grade III malignant schwannoma in an individual without NF1. Immunocytochemical analysis revealed that the overall

pattern of expression for Schwann cell markers is consistent with a Schwann cell origin for each of the four tumor-derived cell lines (Ryan *et al.*, 1994; Klein and De Vries, submitted for publication). Western blotting analysis confirmed that the tumor-derived cell lines expressed significant levels of CNPase (2′, 3′-cyclic nucleotide 3′-phosphohydrolase) and laminin (data not shown). The ST88-14 and T265-2c cell lines displayed high levels of Kit protein compared to the STS-26T and NF1T cells (Figure 1a). Primary rat Schwann cells showed little Kit expression. The NF1 phenotype has been linked to mutations in the *Nf1* gene. Therefore we analysed the expression of neurofibromin in the four cell lines (Figure 1b). Human neurofibromin appeared as a 250 kDa doublet band on Western blots. The schwannoma derived cell line, STS-26T, showed levels of neurofibromin comparable to normal Schwann cells. The two NFS-derived Schwann cell lines expressed very low levels (ST88-14) or no detectable levels of neurofibromin (T265-2c). Surprisingly, NF1 cells, although derived from a patient diagnosed with NF1, displayed levels of neurofibromin comparable to cultured rat Schwann cells or to the STS-26T cell line. Thus, Schwann cell lines expressing neurofibromin did not express appreciable levels of Kit, whereas cell lines deficient in neurofibromin showed high expression of Kit protein.

To evaluate the state of Kit activation in the cell lines expressing high levels of Kit, proteins were immunoprecipitated with an anti-Kit antibody, then analysed using an anti-phosphotyrosine antibody. Two proteins, a 145 kDa protein corresponding to Kit and a smaller protein of approximately 80 kDa, were immunoprecipitated from both NFS-derived Schwann cell lines (Figure 1c, lanes 1 and 2). The astroglioma cell line A172 (lane 3), which has high levels of Kit expression, was used as a positive control. When used in Western blotting analysis of whole cell lysates, the same anti-Kit antibody recognized predominantly the 80 kDa protein. Both Kit and the smaller protein immunologically related to Kit were tyrosyl-phosphorylated (Figure 1d). A 100 kDa phosphotyrosylated protein was also observed, but was immunoprecipitated by non-specific rabbit immunoglobulins as well (lane 4).

Kit expression in the NFS-derived Schwann cells was also analysed by immunocytochemistry (Figure 2a). ST88-14 cells, which can appear as bipolar spindle-shaped cells or pleiomorphic flattened cells, showed high levels of Kit expression throughout the cell bodies and processes. While the level of cytosolic Kit expression was relatively homogeneous within the cell population, some cells also displayed a strong nuclear staining. High expression of Kit was not only observed *in vitro* in the NFS-derived cell lines, but also *in vivo*, as revealed by immunohistochemistry of fresh tissue sections from an NF1 tumor. The tumor, identified as a plexiform neurofibroma, was first analysed using an antibody against the Schwann cell marker, S100. The anti-S100 antibody stained elongated, spindle shaped cells, with a morphology characteristic of the Schwann cells present in neurofibromas (not shown). The Kit antibody had a wider spectrum of staining. In addition to staining the cell bodies and processes of the S100 reactive Schwann cell-like cells, Kit antibody also stained large round cells, which have a morphology consistent with mast cells (Figure 2b).

Kit is present and tyrosyl-phosphorylated in the NF1-derived cell lines. If Kit is involved in the proliferation of the NFS-derived Schwann cells, then inhibition of the tyrosine kinase activity in the cells
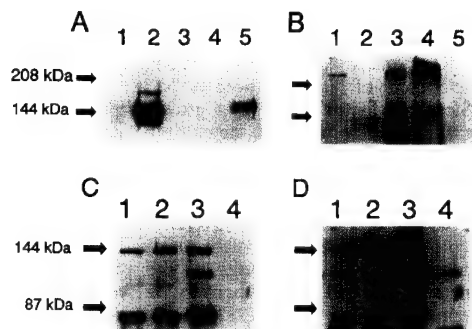
Figure 1 Upper panel: Expression of Kit in NFS-derived cell lines. Primary rat Schwann cells (lane 1), NFS-derived cell lines ST88-14 (lane 2), NF1T (lane 3) and T265-2c (lane 5) and the schwannoma cell line STS-26T (lane 4) were examined for Kit expression (**a**) and neurofibromin expression (**b**). NFS-derived cell lines deficient in neurofibromin (MW 250 kDa) expressed high levels of Kit (MW 145 kDa). Lower panel: Tyrosyl-phosphorylation of Kit in NFS-derived cell lines. Cell lysates from the ST88-14 (lane 1) and T265-2c (lane 2) cell lines and from the glioma cell line A172 (lane 3) were immunoprecipitated with an anti-Kit antibody; lane 4: ST88-14 cell line immunoprecipitated with non-specific rabbit immunoglobulins. Immunoprecipitated proteins were immunoprobed with an anti-Kit antibody (**c**) or an anti-phosphotyrosine antibody (**d**). For immunoprecipitation, cells cultured in serum-free media were lysed and incubated overnight in the presence of a preformed complex of Protein A-agarose/anti-Kit antibody (C-19, Santa-Cruz Biotechnology) at 4°C. The cell lysates and the immunoprecipitated proteins were analysed by electrophoresis in a 7.5% polyacrylamide gel and transfer to a PVDF membrane (Dupong, NEN). After blocking with a 5% nonfat dry milk solution, the PVDF membrane was probed with the appropriate primary antibody (anti-Kit Ab-1 from Oncogene Science, anti-neurofibromin from Santa Cruz Biotechnology or anti-phosphotyrosine from Transduction Laboratories). The antibody was detected using a horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence (Dupont-NEN). Primary rat Schwann cells were prepared according to Brockes and colleagues (1979)
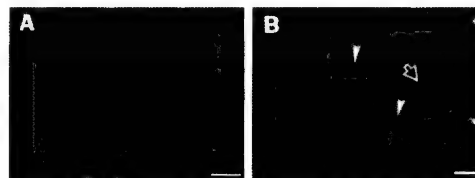
Figure 2 Immunocytochemical detection of Kit in the NFS-derived Schwann cell line ST88-14 (**a**) and in fresh frozen sections (15 μm) of a plexiform neurofibroma (**b**). Cells and tissue were incubated with an anti-Kit antibody (Santa-Cruz Biotechnology) and visualized using a rhodamine-conjugated secondary antibody. Immunocytochemistry was performed as described before (Lee *et al.*, 1996). In the tumor, Kit is present in both large round cells, probably mast cells (arrow) and spindle-shaped, Schwann-like cells with extended processes (arrowheads). In the absence of primary antibody, cells and tissue sections showed no fluorescence. Bar is 10 μm

should decrease their proliferation. The NFS-derived cell lines, ST88-14 and T265-2c, and the A172 line were cultured in the presence of increasing concentrations of tyrosine kinase inhibitors, in serum-free media. Tyrosine kinase inhibitors acting either through competitive inhibition of ATP binding (Genistein) or by competitive inhibition of substrate binding to the kinase domain (Tyrphostin A9 and Tyrphostin A25)

Figure 3 Effect of tyrosine kinase inhibitors on the proliferation of NFS-derived cell lines and the A172 glioma cell line. Cells (5000/well) were incubated in serum-free media for 3 days, in the presence of increasing amounts of Tyrphostin A9 (a), Tyrphostin A25 (b) or Genistein (c), all obtained from LC Laboratories. Cell number at 0 and 3 days was evaluated by the colorimetric MTT assay (Boehringer Mannheim Corporation). Absorbance was measured at 595 nm and growth expressed as the change in absorbance relative to untreated controls. Results were expressed as the mean $\pm$ s.e. of triplicate wells ($n=3-5$). Microscopic examination showed little if any cytotoxicity of the compounds at the doses used in these experiments. (d) ST88-14 cells treated with 5 μM Tyrphostin A9, 150 μM Tyrphostin A25 or 50 μM Genistein as described above were lysed and the proteins analysed by Western blotting using an anti-phosphotyrosine antibody. Densitometric analysis of proteins was performed using the BioRad GS-670 densitometer and expressed relative to control (cells treated with vehicle)

were tested. Tyrphostin A9, a tyrosine kinase inhibitor specific for PDGF-like tyrosine kinase receptors, decreased the rate of NFS-derived Schwann cell proliferation in a dose-dependent manner with an ED50 of approximately 1.5 μM (Figure 3a), close to its Ki/IC$_{50}$ (Levitzki and Gilon, 1991). Tyrphostin A25 and Genistein, with respective ED50 of 80 μM and 25 μM, were efficient inhibitors of cell proliferation only at concentrations largely higher than their published Ki/IC$_{50}$ (Gazit *et al.*, 1989), probably targeting a wide range of tyrosine kinase receptors. To demonstrate that tyrosine phosphorylation was actually inhibited by the tyrosine kinase inhibitors, ST88-14 cells were collected after 3 days of treatment with the tyrosine kinase inhibitors. Proteins were then analysed by Western blotting using an anti-phospho-tyrosine antibody and the lanes were scanned to evaluate the relative amounts of tyrosyl-phosphory-lated proteins. We observed a general decrease in the level of tyrosine phosphorylated proteins in cells cultured in the presence of Genistein (45% decrease), Tyrphostin A9 (85% decrease) and Tyrphostin A25 (90% decrease) which could be correlated to the decrease in cell proliferation observed in the presence of these inhibitors (Figure 3d).

Proliferation of many tumor cell lines is driven by an autocrine loop, involving activation of a growth factor receptor by its ligands expressed by the same cells. To determine if a Kit/SCF autocrine loop was responsible for the NF1 cell line proliferation, cell growth was evaluated in the presence of neutralizing antibodies to SCF (R&D systems) or to Kit (clones 1.D9.3D6, Boehringer Mannheim Corporation or K44.2, Sigma Bioscience). These antibodies have been used to interfere with SCF activity in various systems and in particular to disrupt the autocrine growth of breast carcinoma cell lines (Bleechman *et al.*, 1993; Hines *et al.*, 1995). Anti-SCF antibodies (used at concentrations as high as 50 μg/ml) had no significant effect on the proliferation rate of the NFS-derived Schwann cell lines grown in serum-containing or serum-free media. (Figure 4a). Similarly, both anti-Kit antibodies (used at concentrations as high as 25 μg/ml) failed to prevent the growth of the NFS-derived cell lines (data not

Figure 4 Effect of anti-SCF antibodies (a) and suramin (b) on the proliferation of NFS-derived cell lines and the A172 glioma cell line. Cells were incubated in the presence of increasing amounts of neutralizing antibodies to SCF (R&D Systems) or suramin (Calbiochem) in serum-free media for 3 days and proliferation expressed as in Figure 3. Suramin inhibits the proliferation of the A172 cell line, but has no significant effect on the proliferation of NF1-derived Schwann cell lines

shown). In order to evaluate the possibility of an autocrine loop involving other growth factors and their receptors, cells were cultured in the presence of suramin, a blocker of growth factor/growth factor receptor interactions with a broad range of action. Suramin was very effective in blocking the proliferation of the A172 cell line (Figure 4b), which is known to be largely dependent upon an autocrine loop involving PDGF/PDGF receptors (Fleming et al., 1992; Sato and Nitta, 1994). Suramin did not decrease the growth of the NFS-derived Schwann cell lines. A limited increase in cell number compared to untreated cells was observed at low suramin concentrations (Figure 4b).

In order to directly test if Kit can assume a role in Schwann cell hyperplasia, the effect of SCF on proliferation of the four Schwann cell lines was evaluated (Figure 5a). Cells were cultured in the presence of 20 ng/ml SCF. After 4 days in culture the number of cells was evaluated relative to untreated cells. SCF produced a significant increase in the number of ST88-14 cells. The response of the T265-2c cell line, which expressed intermediate levels of Kit, to SCF was less robust but significant. In contrast, SCF was a poor mitogen for the NF1T and STS-26T cell lines. ST88-14 cells and NF1T cells were cultured over longer periods of time in the presence of increasing concentrations of SCF. SCF stimulated the proliferation of the ST88-14 cells in a dose-dependent manner, leading to a twofold increase in cell number over 10 days, relative to untreated cells (Figure 5b); the effect was maximal at 10 ng/ml and less at 100 ng/ml. SCF did not significantly stimulate the proliferation of the NF1T cell line (Figure 5c).

In this report, we have shown that the proto-oncogene Kit is highly expressed in NFS-derived cells deficient in neurofibromin, but expressed at low levels in primary Schwann cells or malignant Schwann cells with normal levels of neurofibromin expression. Kit expressed by the NFS-Schwann cells is functional and SCF stimulates the NFS-derived Schwann cells to proliferate. These results indicate that high expression of Kit in NFS-derived Schwann cells may be associated with the NF1-phenotype (i.e. neurofibromin deficiency) and that SCF stimulation of Schwann cell proliferation can contribute to the hyperplastic phenotype that leads to Schwann cell tumor formation in NF1 patients.

Kit is expressed at low levels in adult human Schwann cells (Ryan et al., 1994, TJ Lopez and GH DeVries, unpublished data), but is expressed at high levels in the ST88-14 and T265-2c cell lines, two NFS-derived Schwann cell lines. Kit expression appears to be specifically associated with the NF1 phenotype and not, more generally, with a transformed phenotype. Indeed, the STS-26T cell line, a schwannoma-derived cell line from a patient without NF1, and an immortalized rat Schwann cell line (Ryan et al., 1994) display very limited expression of Kit. The observation that the NF1T cell line, which is a NFS-derived cell line, does not display high levels of Kit could appear in contradiction with this hypothesis. But examination of neurofibromin expression in the different cell lines shows that the NF1T cell line, contrary to the other NFS-derived lines, expresses levels of neurofibromin comparable to normal cells. It has been shown that Schwann cell differentiation is
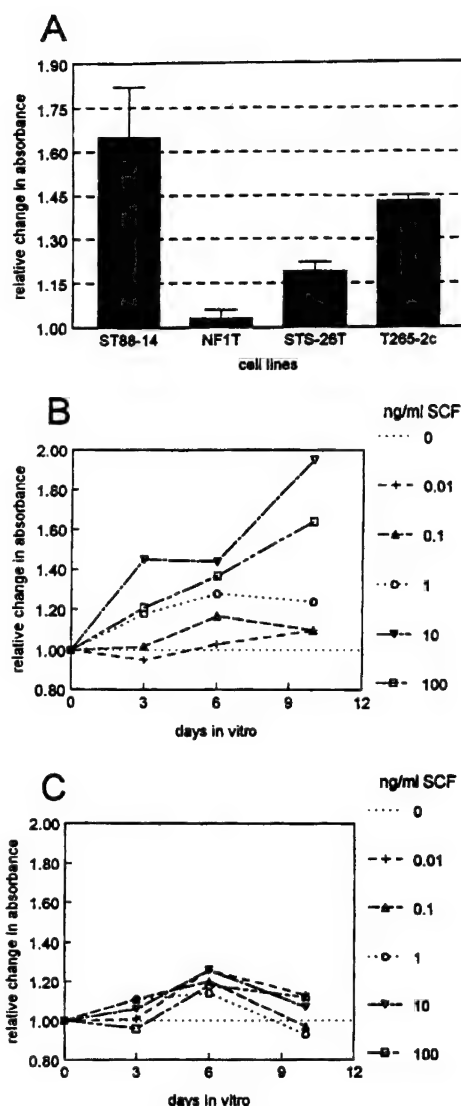
Figure 5 Effect of SCF on the proliferation of the NFS-derived cell lines and the schwannoma-derived cell line. (a) Cell lines were grown in the presence of 20 ng/ml recombinant human SCF (R&D Systems) in serum-free media for 4 days. Cell number was evaluated using an MTT assay and the proliferation expressed relative to untreated cells. ST88-14 cells (b) and NF1T cells (c) were grown in the presence of increasing amounts of SCF in serum-free media, for either 3, 6 or 10 days. Proliferation was expressed relative to untreated cells. Results are expressed as mean of six wells, s.e. did not exceed 10%

associated with increased expression of neurofibromin (Gutmann et al., 1993). Recent studies from our laboratory show that among the four Schwann cell lines described in this study, the NF1T cell line bears the most resemblance to normal Schwann cells. The

NF1T cell line maintains a bipolar morphology characteristic of Schwann cells, has the highest level of CNPase activity (CNPase is a biochemical marker of myelin) and retains its ability to preferentially associate with axons (Klein and DeVries, submitted for publication). Because of its more differentiated phenotype, it is not surprising to observe the expression of neurofibromin in the NF1T cell line. However, we cannot exclude the possibility that neurofibromin expressed in the NF1T cells may present subtle mutations that cannot be resolved using polyacrylamide gel electrophoresis. A recent study described that neurofibromas, with normal levels of neurofibromin, displayed intermediate levels of Ras-GTP, compared to neurofibrosarcomas (Guha *et al.*, 1996). This intermediate phenotype may be insufficient to induce an increase in Kit expression. Thus, although only a limited number of NF1-cell lines have been tested so far, our data indicate that Kit is over-expressed in NFS-derived Schwann cells showing a reduction in neurofibromin. However, a direct relationship between the decrease in neurofibromin and the up-regulation of Kit expression still needs to be demonstrated.

Kit expressed in the NFS-derived cell lines is constitutively tyrosyl-phosphorylated, possibly due to its interaction with endogenously produced SCF. We were able to block the proliferation of the NFS-derived Schwann cell lines, using low doses of an inhibitor selective for the PDGF-receptor subclass of tyrosine kinase receptor. The growth of the NFS-cell lines was not significantly affected by the addition of suramin to the media. Suramin, a polyanionic drug, is thought to interfere with the binding of the growth factors to low affinity, heparan-sulfate proteoglycan receptors. Suramin has been used in many instances to block autocrine growth loops due to PDGF, fibroblast growth factor and insulin-like growth factor, which are all well known mitogens for Schwann cells. It has been shown recently that suramin inhibits the responsiveness of Schwann cells to glial growth factor, the most potent Schwann cell mitogen (Sudhalter *et al.*, 1996). Because of the lack of effect of suramin on the proliferation of the NFS-derived cells, it is unlikely that any of these growth factors contribute to the autocrine growth of the NFS-derived cells. We were unable to interrupt the NFS-derived Schwann cell proliferation using antibodies specific for Kit and SCF. One possibility is that the SCF/Kit interaction takes place intracellularly. Even though the actual mechanism remains unclear, it has been shown that a PDGF/PDGF receptor autocrine growth loop can take place even when PDGF was modified so that it could no longer be secreted extracellularly (Keating and Williams, 1988). It is also believed that a large part of the interactions between SCF and Kit in breast carcinoma cell lines and tumors occurs intracellularly (Hines *et al.*, 1995). It is also possible that the 80 kDa protein represents a truncated, constitutively active form of Kit. Finally, it has recently been shown that, in human astrocytes, high levels of Kit expression in the absence of its ligand resulted in apoptotic cell death (He *et al.*, 1997).

Experiments evaluating the effect of SCF on the Schwann cell lines show unambiguously that Kit activation stimulates the proliferation of the NFS-derived cells. However, the mitogenicity of SCF for the NFS-derived lines, and for the T265-2c cell line in particular, is only moderate. This could be explained by the lower levels of Kit expression of this cell line (relative to the ST88-14) and the fact that a significant proportion of Kit may be interacting with endogenous SCF, limiting the response elicited by exogenous SCF. Similar observations were reported in breast carcinoma cells, co-expressing SCF and Kit; only high levels of Kit expression allow a growth response to an excess of SCF (Hines *et al.*, 1995). The dose required to elicit maximal cell growth in our study is relatively low, possibly due to the fact that Kit binding sites are already partially occupied by endogenous SCF. In the hemopoietic system, SCF alone has a very modest effect on the proliferation of early hemopoietic precursors but it markedly potentiates the effect of other growth factors, such as interleukin 3, erythro-poietin, GM-CSF and G-CSF (McNiece *et al.*, 1991). Thus, it is possible that SCF may act in cooperation with other factors to stimulate NFS-derived Schwann cell proliferation. In that context, it will be interesting to test the effect of factors that increase intracellular cyclic AMP levels, as elevating cyclic AMP levels in *Nf1*-deficient mouse Schwann cells results in Schwann cell hyperplasia (Kim *et al.*, 1997).

We have shown that Kit is expressed at high levels in NFS-derived cells displaying decreased neurofibro-min expression and that Kit activation by SCF can induce a proliferative response in NFS-derived cells. In the tumors, Kit may be activated by endogenous SCF produced by the Schwann cell-like cells themselves. However, *in vivo*, exogenous sources of SCF such as the fibroblasts present in the tumors and circulating SCF from the serum can contribute to Kit activation. The respective contributions of the various sources of SCF to Schwann cell hyperplasia in the neurofibro-sarcomas still need to be investigated, as does the possible involvement of co-factors, produced by other cell types present in the tumors (fibroblasts, mast cells) which could potentiate SCF mitogenicity towards Schwann cells. In turn, a better understanding of the cellular mechanisms leading to the abnormal prolifera-tion of Schwann cells in neurofibromas and neurofi-brosarcomas will allow the design of new therapeutic strategies for NF1.

References

Basu TN, Gutmann DH, Fletcher JA, Glover TW, Collins FS and Downward J. (1992). *Nature*, **356**, 713–715.

Bleechman JM, Lev S, Leitner O, Givol D and Yarden Y. (1993). *J. Biol. Chem.*, **268**, 4399–4406.

Brockes JP, Fields KL and Raff MC. (1979). *Brain Res.*, **165**, 105–108.

Buchberg AM, Cleveland LS, Jenkins NA and Copeland NG. (1990). *Nature*, **347**, 291–294.

Cawthon RM, Weiss R, Xu G, Viskochil D, Culver M, Stevens J, Robertson M, Dunn D, Gesterland R, O'Connel P and White R. (1990). *Cell*, **62**, 193–201.

Datson MM, Scrable H, Nordlund M, Sturbaum AK, Nissen LM and Ratner N. (1992). *Neuron*, **8**, 415–428.

DeClue JE, Papageorge AG, Fletcher JA, Diehl SR, Ratner N, Vass WC and Lowy DR. (1992). *Cell*, **69**, 265–273.

Ducatman BS, Scheithauer BW, Piepgras DG and Reiman HM. (1986). *Cancer*, **57**, 2006–2021.

Fleming TP, Matsui T, Heidaran MA, Molloy CJ, Artrip J and Aaronson SA. (1992). *Oncogene*, **7**, 1355–1359.

Gazit A, Yaish P, Gilon C and Levitzi A. (1989). *J. Med. Chem.*, **32**, 2344–2352.

Guha A, Lau N, Huvar I, Gutmann D, Provias J, Pawso and Boss G. (1996). *Oncogene*, **12**, 507–513.

Gutmann DH, Tennekoon GI, Cole JL, Collins FS and Rutkowski JL. (1993). *J. Neurosci. Res.*, **36**, 216–223.

Halling KC, Scheithauer BW, Halling AC, Nascimento AG, Ziesmer SC, Roche PC and Wollan PC. (1996). *Am. J. Clin. Pathol.*, **106**, 282–288.

He J, DeCastro CM, Vandenbark GR, Busciglio J and Gabudza D. (1997). *Proc. Natl. Acad. Sci. USA*, **94**, 3954–3959.

Hines SJ, Organ C, Kornstein MJ and Krystal GW. (1995). *Cell Growth and Differ.*, **6**, 769–779.

Keating MT and Williams LT. (1988). *Science*, **239**, 914–916.

Kim HA, Ling B and Ratner N. (1997). *Mol. Cell. Biol.*, **17**, 862–872.

Kim HA, Rosenbaum T, Marchionni MA, Ratner N and DeClue JE. (1995). *Oncogene*, **11**, 325–335.

Lee MMS, Sato-Bigbee C and De Vries GH. (1996). *J. Neurosci. Res.*, **46**, 204–210.

Levitzki A and Gilon C. (1991). *TIPS*, **12**, 171–174.

McNiece I, Langley K and Zsebo K. (1991). *Exp. Hematol.*, **19**, 226–231.

Riccardi VM. (1991). *N. Engl. J. Med.*, **324**, 1283–1285.

Ridley AJ, Paterson HF, Noble M and Land H. (1988). *EMBO J.*, **7**, 1635–1645.

Ryan JJ, Klein KA, Neuberger TJ, Leftwich JA, Westin EH, Kauma S, Fletcher JA, De Vries GH and Huff TF. (1994). *J. Neurosci. Res.*, **37**, 415–432.

Sato MD and Nitta K. (1994). *Neurosurgery*, **34**, 309–315.

Sudhalter J, Whitehouse L, Rusche JR, Marchionni MA and Mahanthappa NK. (1996). *Glia.*, **17**, 28–38.

Viskochil D, Buchberg AM, Xu G, Cawthon RM, Stevens J, Wolf RK, Culver M, Carey JC, Copeland NG, Jenkins NA, White P and O'Connell P. (1990). *Cell*, **62**, 187–192.

Wallace MR, Marchuk DA, Andersen LB, Letcher R, Odeh HM, Saulino AM, Fountain JW, Brereton A, Nicholson J, Mitchell AL, Brownstein BH and Collins FS. (1990). *Science*, **249**, 181–186.

Williams DE, de Vries P, Namen AE, Widmer MB and Lyman SD. (1992). *Dev. Biol.*, **151**, 368–376.

Xu G, O'Connell P, Viskochil D, Cawthon RM, Robertson M, Culver M, Dunn D, Stevens J, Gesteland R, White P and Weiss R. (1990). *Cell*, **62**, 599–608.

Yarden Y, Kuang W, Yang-Fen T, Coussens L, Munemitsu S, Dull TL, Chen E, Schlessinger JL, Franckle U and Ullrich A. (1987). *EMBO J.*, **6**, 3341–3351.

Neurofibrosarcoma-Derived Schwann Cells Overexpress Platelet-Derived Growth Factor (PDGF) Receptors and Are Induced to Proliferate By PDGF BB

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Neurofibromatosis type 1 (NF1) is characterized by the formation of neurofibromas, benign tumors of the peripheral nerve consisting essentially of Schwann cells, which can sometimes turn malignant to form neurofibrosarcomas. The mechanism of progression toward a malignant phenotype remains largely unknown. In this report, we show that platelet-derived growth factor (PDGF) BB, and to a lesser extent fibroblast growth factor 2, are mitogenic for two neurofibrosarcoma-derived Schwann cell lines, but not for a Schwann cell line derived from a schwannoma (from a non-NF1 patient) or for transformed rat Schwann cells. Levels of expression of both PDGF receptor α and β are significantly increased in the two neurofibrosarcoma-derived cell lines compared to the non-NF1 Schwann cell lines. The level of tyrosyl-phosphorylated PDGF receptor β is strongly increased upon stimulation by PDGF BB. In comparison, only modest levels of tyrosyl-phosphorylated PDGF receptor α are observed, upon stimulation by PDGF AA or PDGF BB. Accordingly, PDGF AA is only a weak mitogen for the neurofibrosarcoma-derived cells by comparison to PDGF BB. These results indicate that the mitogenic effect of PDGF BB for the neurofibrosarcoma-derived Schwann cell lines is primarily transduced by PDGF receptor β. Neu differentiation factor β, a potent mitogen for normal Schwann cells, was unable to stimulate proliferation of the transformed Schwann cell lines, due to a dramatic downregulation of the erbB3 receptor. Therefore, aberrant expression of growth factor receptors by Schwann cells, such as the PDGF receptors, could represent an important step in the process leading to Schwann cell hyperplasia in NF1. J. Cell. Physiol. 177:334–342, 1998. © 1998 Wiley-Liss, Inc.

Neurofibromatosis type 1 (NF1) is a common inherited disease affecting one in 3,000 individuals, which manifests with various symptoms including neurofibromas, café-au-lait spots, axillary freckling, bone dysplasia, and learning disabilities (Riccardi et al., 1991). Neurofibromas are benign tumors of the peripheral nerve sheath, composed primarily of Schwann cells, but also fibroblasts, perineurial cells, and mast cells. NF1 patients are at higher risk of developing malignant peripheral nerve sheath tumors, also called neurofibrosarcomas, which may arise from neurofibromas. The defective gene in NF1, identified by positional cloning (Cawthon et al., 1990; Viskoshil et al., 1990; Wallace et al., 1990), codes for a protein called neurofibromin, which shares sequence homology with GTPase-activating proteins (Buchberg et al., 1990; Xu et al., 1990). It was predicted that the loss of neurofibromin would result in increased Ras activity that could lead to Schwann cell hyperplasia (Basu et al., 1992; DeClue et al., 1992). Recent studies confirmed that Ras-GTP levels are elevated in neurofibrosarcomas from NF1 patients (Guha et al., 1996) and in Schwann cells derived from *Nf1* mutant mouse (Kim et al., 1995). Nevertheless the actual mechanisms leading to the formation of neurofibromas and neurofibrosarcomas remains unclear. Both epigenetic events and multiple genetic changes have been proposed to contribute to the formation of the peripheral nerve sheath tumors in NF1 (Rosenbaum et al., 1997).

Overexpression of growth factors and/or their receptors is believed to play an important role in cellular transformation. Neurofibromas, which fail to grow when implanted under the skin of nude mice, do grow when implanted into the peripheral nerve, implicating

nerve growth factors in tumor growth (Lee et al., 1992). However, the growth factors and growth factor receptors promoting neurofibromas and/or neurofibrosarcomas growth remain unknown. We have shown that, in contrast to normal Schwann cells or non-NF1 transformed Schwann cells, neurofibrosarcoma-derived Schwann cell lines expressed high levels of Kit, the tyrosine kinase receptor for stem cell factor (SCF) (Ryan et al., 1994; Badache et al., 1998). Moreover, SCF was able to stimulate the proliferation of neurofibrosarcoma-derived lines. Since the effect of SCF on neurofibrosarcoma-derived Schwann cells was only moderate, we investigated the possibility that, in a manner similar to what has been described in the hematopoietic system (McNiece et al., 1991), SCF has a modest mitogenic effect on its own but may act synergistically with other factors to induce cell proliferation. Several potential co-factors for SCF were tested, including neu-differentiation factor β (NDFβ), a member of a family of growth factors called neuregulins, which are potent mitogens for both human and rat Schwann cells (Marchionni et al., 1992; Levi et al., 1995); platelet-derived growth factor BB (PDGF BB) and fibroblast growth factor 2 (FGF2), well-known mitogens for rat Schwann cells (Davis and Stroobant, 1990); and forskolin, an agent which increases the levels of intracellular cAMP, known to potentiate the mitogenicity of FGF2, PDGF BB, and NDFβ, by inducing increased expression of their receptors (Weinmaster and Lemke, 1990; Cohen et al., 1990).

SCF had no synergistic effect on cell proliferation when combined with either NDFβ, PDGF BB, FGF2, or forskolin. However, PDGF BB and to a lesser extent FGF2 appeared to be strongly mitogenic on their own. NDFβ, the most potent mitogen for normal Schwann cells, had no effect on neurofibrosarcoma-derived Schwann cell proliferation. Further studies revealed drastic changes in the pattern of growth factor receptors expressed by the neurofibrosarcoma-derived Schwann cells. Our results reveal that growth factors, PDGF BB in particular, may play an significant role in the aberrant growth of Schwann cells that leads to tumor formation in NF1.

MATERIAL AND METHODS
Cell lines and growth factors

The ST88-14 cell line (obtained from Jonathan Fletcher, Brigham and Women Hospital, Boston, MA) and the T265-2c cell line (developed by Karen Klein in our laboratory) were derived from malignant peripheral nerve tumors resected from patients with NF1. The STS-26T was derived from an isolated grade III malignant Schwannoma in an individual without NF1 and was obtained from William Dahlberg (Harvard School of Public Health, Boston, MA). The RSC-96 line is a spontaneously transformed rat Schwann cell line derived from long term culture of rat primary Schwann cells. Cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS; HyClone, Logan, UT). All the cell lines express the Schwann cell markers S100, CNPase, and laminin, as seen by immunocytochemistry and/or immunoblotting. Recombinant human PDGF AA, PDGF BB, FGF2, and SCF were obtained from R&D Systems (Minneapolis, MN). Recombinant NDFβ1 (amino-acids

14-241) was a gift from Amgen (Thousand Oaks, CA). Forskolin was from Calbiochem (La Jolla, CA).

Proliferation assay

To measure cell proliferation, cells were replated in 96-well clusters at 5,000 cells/well. Cells were allowed to adhere to the substratum in DMEM containing 10% FCS and then switched to serum-free DMEM, containing the growth factors at the doses indicated in the text. Media and growth factors were replenished every 3 days. Cell number was evaluated by using the colorimetric MTT assay (Boehringer-Mannheim, Indianapolis, IN) following the manufacturer's protocol. Absorbance was measured at 595 nm and was shown to correlate with viable cell number under the conditions used in this study.

Western blotting and immunoprecipitation

Cells were lysed in RIPA buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate, and SDS, in phosphate buffered saline, PBS, pH 7.2) containing 1 mM sodium orthovanadate and a cocktail of protease inhibitors. Protein concentration was determined by the DC protein assay (Bio-Rad, Hercules, CA). For immunoprecipitation experiments, cells incubated in serum-free medium in the presence of 50 ng/ml PDGF AA or PDGF BB for 15 min were lysed in RIPA buffer. Equivalent amounts of protein were incubated at 4°C for 2 h in the presence of a preformed complex of Protein A/G-Agarose (Santa-Cruz Biotechnology, Santa Cruz, CA) and an antibody specific for PDGF receptor α or β (Oncogene Product, Cambridge, MA). After several washes, the immunoprecipitated proteins were separated by electrophoresis in a 7.5% SDS-polyacrylamide gel and transferred to a PVDF membrane (Dupont NEN, Boston, MA). After blocking with a 5% non-fat dry milk solution, the PVDF membrane was incubated for 2 h in the presence of the primary antibody (anti-PDGF receptor α, anti-PDGF receptor β, anti-erbB2, anti-erbB3 from Santa Cruz Biotechnology, anti-Kit from Oncogene Product, or anti-phosphotyrosine from Transduction laboratories, Lexington, KY). After several washes in PBS containing 1% Tween-20, the membrane was incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA) and the immunoreactivity detected by enhanced chemiluminescence (Dupont-NEN, Boston, MA).

RESULTS
Effect of growth factors on neurofibrosarcoma-derived cell proliferation

The effect of SCF, NDFβ, PDGF BB, and FGF2 on the growth of two neurofibrosarcoma-derived Schwann cell lines was evaluated; the ST88-14 cell line and the T265-2c cell line are neurofibromin-deficient Schwann cell lines, derived from neurofibrosarcomas from NF1 patients (Badache et al., 1998). Both cell lines expressed the Schwann cell markers S100, CNPase, and laminin as seen by immunocytochemistry and/or immunoblotting (Ryan et al., 1994; Badache, Klein and DeVries, unpublished observations). NDFβ, PDGF BB, and FGF2 are known mitogens for rat Schwann cells (Davis and Stroobant, 1990; Raabe et al., 1997). NDFβ

is the only potent mitogen for human Schwann cells (Levi et al., 1995; Lopez and DeVries, unpublished observations). Cells were cultured in serum-free medium in the presence of 20 ng/ml growth factor and cell number was evaluated after 4, 7, and 10 days after treatment. SCF had a moderate effect on ST88-14 cell proliferation, as shown before (Fig. 1A). Surprisingly NDFβ mitogenicity for the ST88-14 cell line was only modest and comparable to SCF effect. On the other hand, PDGF BB, and to a lesser extent FGF2, which are not mitogens for human Schwann cells (Li et al., 1996), elicited a strong mitogenic response from the ST88-14 cells. After 7 days in culture, the rate of cell growth was slower, most probably due to cell death due to culturing the cells in the serum-free medium. Similar results were obtained using the T265-2c cell line (Fig. 1B): PDGF BB and FGF2 were strong mitogens for these cells, whereas SCF was a weak mitogen. NDFβ did not stimulate proliferation of the T265-2c cells. Similar experiments were performed using Schwann cell lines, not derived from NF1 patients. We used the spontaneously transformed rat cell line RSC-96 and a Schwann cell line derived from a Schwannoma in a non-NF1 patient, called STS-26T. SCF is not a mitogen for the STS-26T cell line (as we have previously shown, Badache et al., 1998), nor for the RSC-96 line (Fig. 1C,D). NDFβ, FGF2, and PDGF BB also failed to stimulate significantly the growth of the two non-NF1 Schwann cell lines even after 10 days in culture (Fig. 1C,D).

PDGF BB and FGF2 induced the proliferation of the ST88-14 cell line (data not shown) and the T265-2c cell line in a dose-dependent manner (Fig. 2A,B): both PDGF BB and FGF2 significantly stimulated cell growth at doses as low as 0.1 to 1 ng/ml and had a maximal effect around 50 ng/ml. Higher doses of PDGF BB or FGF2 did not further increase the rate of proliferation. When cultured in the presence of 50 ng/ml PDGF BB or FGF2, over a period of five days, T265-2c cell number was increased approximately two-fold and five-fold by FGF2 and PDGF BB, respectively.

To investigate whether, similarly to what is observed in the hematopoietic system (McNiece et al., 1991), SCF can synergize with other growth factors to promote Schwann cell proliferation, we have evaluated the effect of SCF in combination with PDGF BB, FGF2, or NDFβ on neurofibrosarcoma-derived Schwann cell proliferation. SCF did not significantly influence the mitogenicity of PDGF BB, FGF2, or NDFβ for the T265-2c cells (Fig. 3A) or the ST88-14 cell line (not shown) over the period of time investigated. Combination of SCF with NDFβ, PDGF BB or FGF2 did not induce a mitogenic response of the schwannoma-derived cell line, STS26T (Fig. 3B) or the rat cell line RSC-96 (data not shown).

Agents that increase intracellular cAMP, like forskolin, strongly potentiate the mitogenicity of most Schwann cell growth factors including FGF2, PDGF BB, and neuregulins (Davis and Stroobant, 1990; Goodearl et al., 1993; Levi et al., 1995). Therefore, growth factor induced-proliferation of the neurofibrosarcoma-Schwann cells was also evaluated in the presence of 5 µM forskolin (Fig. 3A). Surprisingly, forskolin had no synergistic effect on the mitogenicity of SCF, NDFβ, FGF2, or PDGF BB or combinations of SCF with

the other growth factors. Proliferation of the neurofibrosarcoma-derived Schwann cell lines in the presence or absence of growth factors was significantly decreased by forskolin over 7 days. Over longer period of times forskolin appeared cytotoxic to the cells. Interestingly, PDGF BB and to a lesser extent FGF2 were able to protect the cells from forskolin-induced cell death. Similarly to what we observed for the neurofibromin-deficient cell lines, forskolin did not potentiate the effect of the growth factors on the RSC-96 and STS-26T cells (Fig. 3B). However, forskolin appeared less cytotoxic to the latter. It is noteworthy that the RSC-96 and STS-26T were able to maintain a sustained proliferation rate in the absence of serum, over the period of time investigated (not shown).

Thus, PDGF BB and to a lesser extent FGF2 are strong mitogens for neurofibrosarcoma-derived Schwann cell lines. By comparison, NDFβ and SCF are only weak mitogens. Forskolin did not potentiate the mitogenicity of any of the growth factors studied for the neurofibrosarcoma-derived Schwann cells.

Growth factor receptors in the neurofibrosarcoma-derived Schwann cell lines

We then investigated whether the responsiveness of neurofibrosarcoma-derived Schwann cells to PDGF but not to NDFβ could be explained by changes in expression of the receptors for these growth factors. We examined expression of the receptors for PDGF (namely PDGF receptor α and PDGF receptor β), for NDFβ (erbB2 and erbB3) and SCF (Kit) in the various cell lines, by immunoblotting. PDGF receptor α is expressed at high levels only in the ST88-14 and T265-2c cell lines (Fig. 4A). It is virtually absent in the STS-26T cell line and expressed at low levels in the RSC-96 lines. Similarly, PDGF receptor β was highly expressed only in the two neurofibromin-deficient cell lines (Fig. 4A). PDGF receptor β was expressed at very low levels in the STS-26T cell line. We also confirmed that Kit was highly expressed only in the ST88-14 and T265-2c Schwann cell lines (Fig. 4A), as we have previously described.

ErbB2, the heterodimerization partner of NDFβ receptor erbB3, was expressed in all cell lines but the schwannoma-derived STS-26T cell line (Fig. 4B). Contrary to what was observed in primary Schwann cells, erbB3 was not expressed at detectable levels in the tumor-derived Schwann cell lines (Fig. 4B).

Activation of PDGF receptors by PDGF BB in the T265-2c cells

To measure the activation of PDGF receptors activation upon PDGF BB stimulation, T265-2c cells were cultured in the presence of 20 ng/ml PDGF BB for increasing periods of time (from 1 min to 2 h) and the levels of tyrosyl-phosphorylated PDGF receptors analyzed by immunoblotting, by using an anti-phosphotyrosine specific antibody (Fig. 5). Tyrosyl-phosphorylation of the receptor was significantly increased (three- to four-fold over basal levels) less than 1 min after stimulation by PDGF BB and reached a maximum (corresponding to an average ten-fold increase over basal level) after 10–15 min of stimulation. Tyrosyl-phosphorylation was down to basal levels within 2 h. The changes in tyrosyl-phosphorylation were not due to

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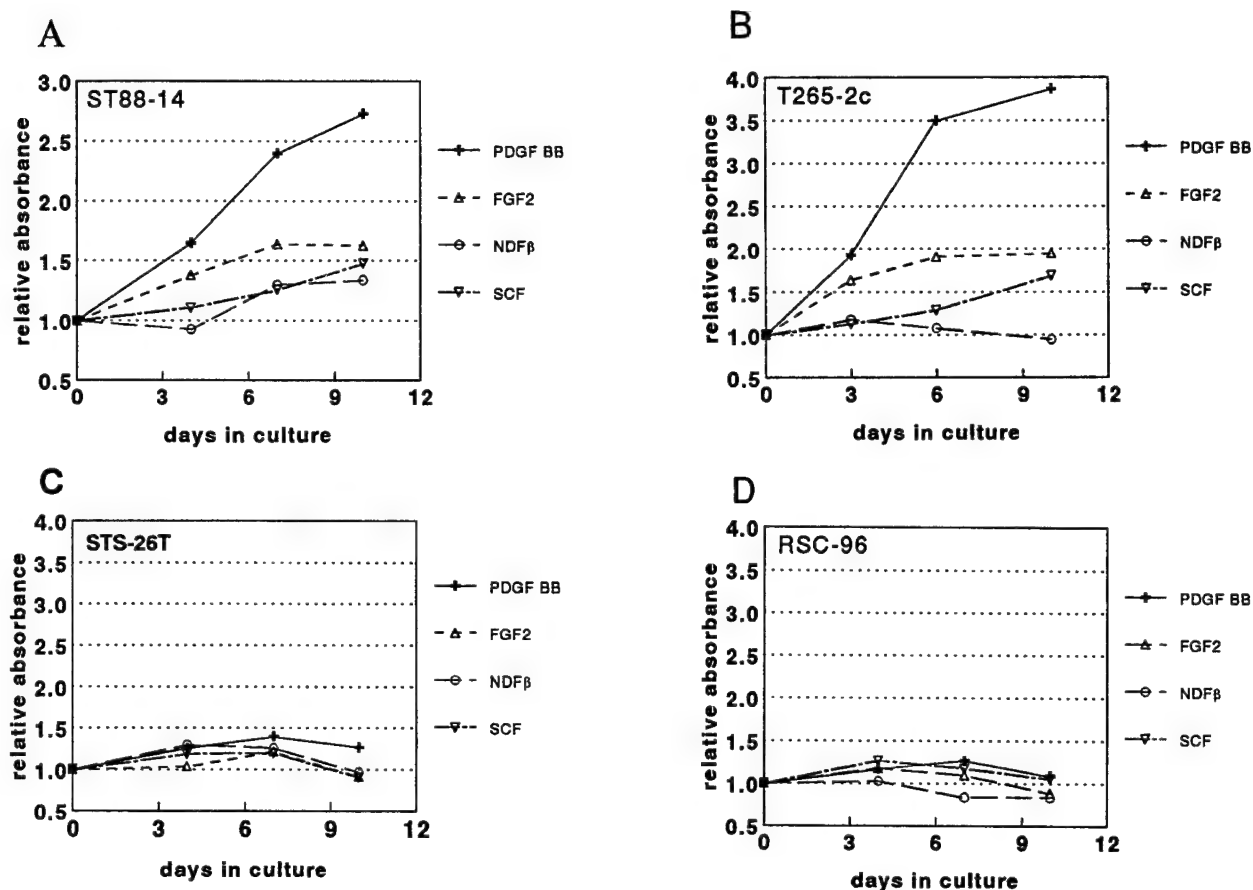
Fig. 1. PDGF BB and FGF2 are mitogenic for neurofibrosarcoma-derived Schwann cells. The neurofibrosarcoma-derived cell lines ST88-14 (**A**) and T265-2c (**B**), the schwannoma-derived cell line STS26T (**C**) and the rat Schwann cell line RSC-96 (**D**) were cultured in serum-free medium in the presence of 20 ng/ml PDGF BB, FGF2 or SCF or 50 ng/ml NDFβ. Cell number was evaluated over 10 days by the colorimetric MTT assay and expressed relative to untreated cells. Values are the results of four to six replicates from a representative experiment repeated two (C and D) or three times (A and B). Standard deviation did not exceed 10%.

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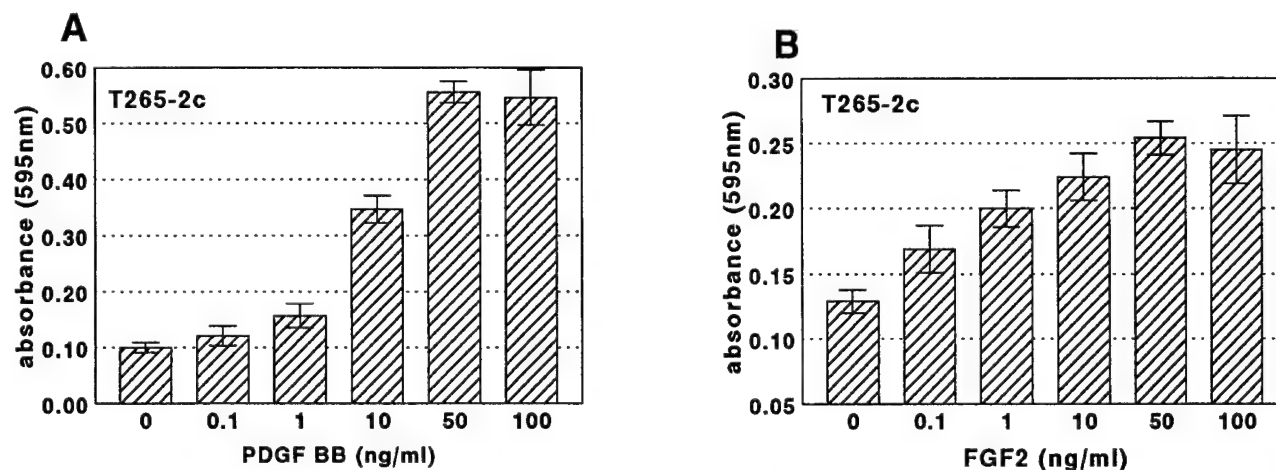
Fig. 2. PDGF BB- and FGF2-induced proliferation of neurofibrosarcoma-derived Schwann cell is dose-dependent. T265-2c cells were cultured in serum-free medium in the presence of increasing amount of PDGF BB (**A**) or FGF2 (**B**). Cell number was evaluated by the colorimetric MTT assay after 6 days in culture. Values are expressed as mean +/− standard deviation of six replicates from a representative experiment repeated at least three times.

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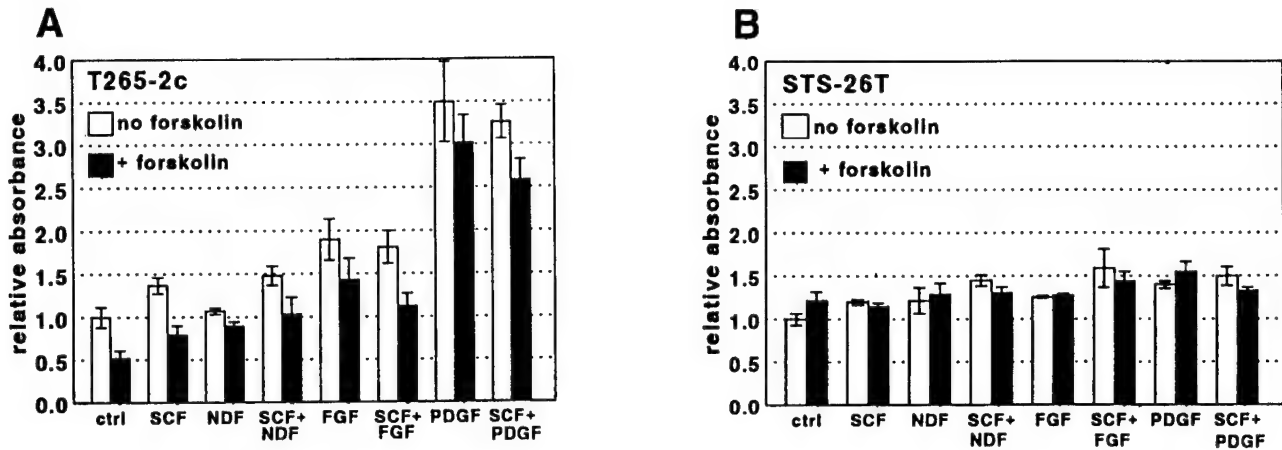
Fig. 3. Effect of NDFβ, FGF2, PDGF BB and forskolin on SCF-induced Schwann cell lines proliferation. The neurofibrosarcoma-derived cell line, T265-2c (**A**) and the schwannoma-derived STS-26T (**B**) were cultured in serum-free medium for 6 days, in the presence of 20 ng/ml SCF combined with 50 ng/ml NDFβ, 20 ng/ml FGF2, or 20 ng/ml PDGF BB in the absence (white bars) or presence (dark bars) of 5 μM forskolin. Cell number was evaluated as described in Figure 2.

changes in the levels of PDGF receptor expression. Indeed, levels of PDGF receptor β and PDGF receptor α remained unchanged within the first 30 min of the experiment, as seen after reprobing of the membrane with antibodies specific for the PDGF receptors (Fig. 5, middle and lower panel). However, after 2 h of stimulation the levels of both PDGF receptors were significantly down-regulated.

Effect of PDGF AA on T265-2c cell proliferation

The neurofibrosarcoma-derived Schwann cells, contrary to normal Schwann cells, displayed high levels of PDGF receptor α and β expression. It is known that PDGF BB can bind with high affinity to PDGF receptor α and to PDGF receptor β. Therefore PDGF BB could induce mitogenic signals through either receptor. PDGF AA, however, is known to bind and activate PDGF receptor α specifically. It was therefore of interest to evaluate the mitogenicity of PDGF AA for the neurofibrosarcoma-derived cell lines. T265-2c cells were cultured in serum-free media in the presence of 50 ng/ml PDGF AA or PDGF BB for 3 to 9 days. PDGF BB induced very robust growth of the T265-2c cells (Fig. 6A). Comparatively, PDGF AA had only a weak mitogenic effect on T265-2c cells. The growth rate of the PDGF AA-stimulated cells was approximately three times lower than the growth rate of the PDGF BB-stimulated cells. PDGF BB-induced proliferation was dose dependent (Fig. 6B), with a maximum effect observed at 50 ng/ml, as shown before. PDGF AA dose-response analysis revealed a plateau between 0.1 ng/ml and 10 ng/ml PDGF AA and a second slightly higher plateau above 50 ng/ml (Fig. 6B).

To understand why PDGF AA mitogenicity for T265-2c cells was only moderate, we analyzed PDGF AA-induced activation of PDGF receptors (Fig. 6C). Levels of tyrosyl-phosphorylated PDGF receptors induced by PDGF AA were very low when compared to PDGF BB-induced tyrosyl-phosphorylation. Upon stimulation with PDGF AA, the level of activated PDGF receptors was increased within 1 min and back to baseline within 30 min.

The previous results indicated that PDGF BB mitogenic activity is transduced by PDGF receptor β. To find out unambiguously which PDGF receptor is activated by PDGF AA or PDGF BB, immunoprecipitation experiments were performed using specific antibodies for PDGF receptor α or β (Fig. 7). T265-2c cells, incubated with 50 ng/ml of PDGF AA or PDGF BB for 10 min, were immunoprecipitated with an antibody to PDGF receptor α or to PDGF receptor β and the level of tyrosyl-phosphorylated PDGF receptor analyzed by immunoblotting. PDGF BB-induced cells expressed high levels of tyrosyl-phosphorylated PDGF receptor β, but only low levels of activated PDGF receptor α (Fig. 7, upper panel). PDGF AA induced the formation of low levels of activated PDGF receptor α, but no tyrosyl-phosphorylated PDGF receptor β, confirming the specificity of PDGF AA for the α receptor and providing an explanation for the modest mitogenicity of PDGF AA. Differences in the levels of tyrosyl-phosphorylated proteins were actually due to changes in the levels of phosphorylation and not to differences in the amount of protein immunoprecipitated (Fig. 7, lower panel).

DISCUSSION

Despite the identification of the *Nf1* gene as a primary target in NF1, the mechanisms leading to the formation of neurofibromas and neurofibrosarcomas remain unclear. In this study, we show that two neurofibrosarcoma-derived Schwann cell lines display radically modified growth factor receptor expression compared to Schwann cell lines not derived from NF1 patient or to adult Schwann cells (Hardy et al., 1992; Eccleston et al., 1993), resulting in functional changes in their mitogenic response to growth factors. PDGF BB induces a strong proliferative response from the two neurofibrosarcoma-derived Schwann cell lines. Mitogenicity of PDGF BB is transduced essentially through PDGF receptor β, which is highly increased in these two cell lines, compared to non-NF1 Schwann cell lines. PDGF receptor α expression is also increased in the neurofibrosarcoma-derived Schwann cells, but does not seem to play an important role in neurofibrosar-
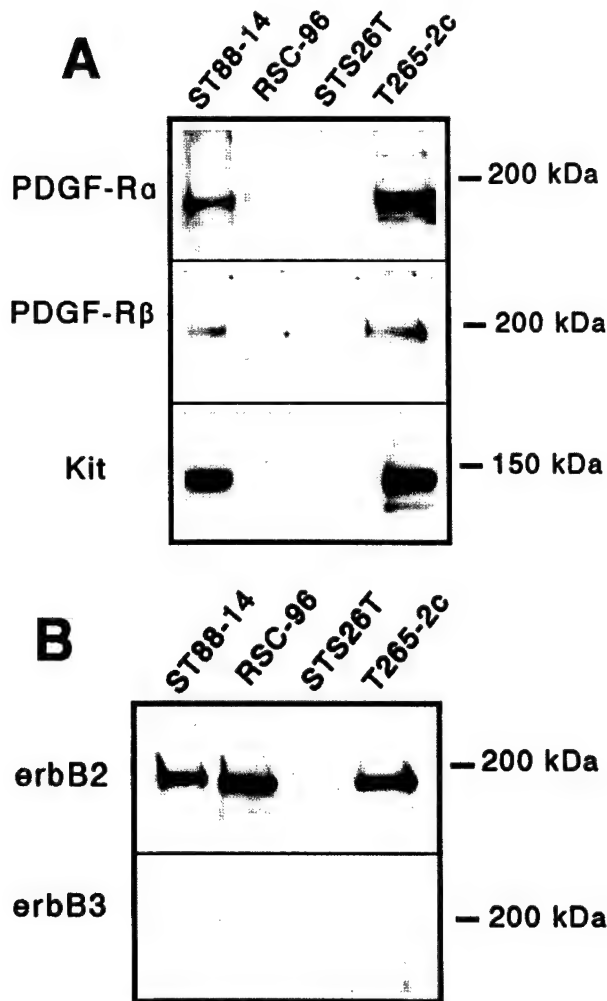
Fig. 5. Time-course of PDGF BB-induced activation of PDGF receptors. T265-2c cells were stimulated with 50 ng/ml PDGF BB. At the indicated time points, cells were lysed and proteins analyzed by immunoblotting using antibodies specific for phosphotyrosine (**upper panel**). Levels of PDGF receptors were estimated by probing the membrane with antibodies to PDGF receptor β (**middle panel**) and PDGF receptor α (**lower panel**).

Fig. 4. Expression of PDGF receptors, Kit and erbB2/3 receptors in neurofibrosarcoma-derived Schwann cell lines. Cell lysates (50 μg/lane) from neurofibrosarcoma-derived Schwann cell lines (ST88-14 and T265-2c), the schwannoma-derived cell line STS-26T, and the rat Schwann cell line RSC-96 were analyzed by immunoblotting by using antibodies specific for PDGF receptor α (**A, upper panel**), PDGF receptor β (A, **middle panel**), Kit (A, **lower panel**), erbB2 (**B, upper panel**), or erbB3 (B, **lower panel**).

coma-derived Schwann cell proliferation. Indeed, the low levels of activated PDGF receptor α observed upon PDGF AA (or PDGF BB) stimulation are insufficient to induce an effective proliferative response from the neurofibrosarcoma-derived Schwann cells. FGF2 also appears to be mitogenic for the neurofibrosarcoma-derived Schwann cells, whereas NDFβ has no effect on neurofibrosarcoma-derived Schwann cell proliferation, due to a dramatic decrease in erbB3 expression.

PDGF BB and FGF2 are known to be potent mitogens for rat Schwann cells, especially in the presence of elevated intracellular cAMP (Davis and Stroobant, 1990), but are not known to induce proliferation of human Schwann cells (Li et al., 1996). Indeed, neuregulins are the only potent mitogens for human Schwann cells (Levi et al., 1995). Neurofibrosarcoma-derived Schwann cells proliferate in response to PDGF BB and FGF2 but are

not affected by the addition of NDFβ, a neuregulin isoform which is a potent mitogen for cultured rat and human Schwann cells (Raabe et al., 1997; Lopez and De Vries, unpublished observations). This radical change in responsiveness to the different growth factors can be readily explained, at least in the case of PDGF BB and NDFβ, by a change in the repertoire of growth factor receptors expressed by the neurofibrosarcoma-derived Schwann cells. The NF1-derived cell lines overexpress PDGF receptors compared to a Schwannoma-derived cell line or a rat Schwann cell line, cultured in the same conditions. Studies of PDGF receptor expression during sciatic nerve development show that, while PDGF receptor β is expressed at relatively high levels in neonatal Schwann cells, it is expressed at low levels in the adult. There is little or no expression of PDGF receptor α in adult Schwann cells (Hardy et al., 1992; Eccleston et al., 1993). Therefore inappropriate expression of PDGF receptors, especially PDGF receptor β, recapitulates an early step of development corresponding to active proliferation of Schwann cells.

NDFβ does not stimulate the growth of the neurofibrosarcoma-derived Schwann cell lines due to the loss of the high affinity neuregulin receptor, erbB3. Only the schwannoma-derived cell line shows a strong decrease in erbB2 receptor expression. Actually, erbB2 has no known ligand but acts as preferred heterodimerization partner for erbB3 (Tzahar et al., 1997). ErbB3 has high affinity for neuregulins, but by itself is devoid of kinase activity (Guy et al., 1994). Upon binding to neuregulin, erbB2/erbB3 form a complex with high ligand affinity and potent signaling activity. Thus, the loss of one of the two partners in the erbB2/erbB3 complex (in this case the loss of erbB3) is sufficient to prevent NDFβ-induced activity. Changes in neuregulin receptor expression do not seem to be related to NF1 specifically, since the schwannoma-derived cell line and the rat transformed cells also show strongly reduced erbB3 expression. Decreased erbB3 expression may therefore represent a general step in the process of Schwann cell malignant transformation. It remains unclear why a molecule which is central for Schwann cell

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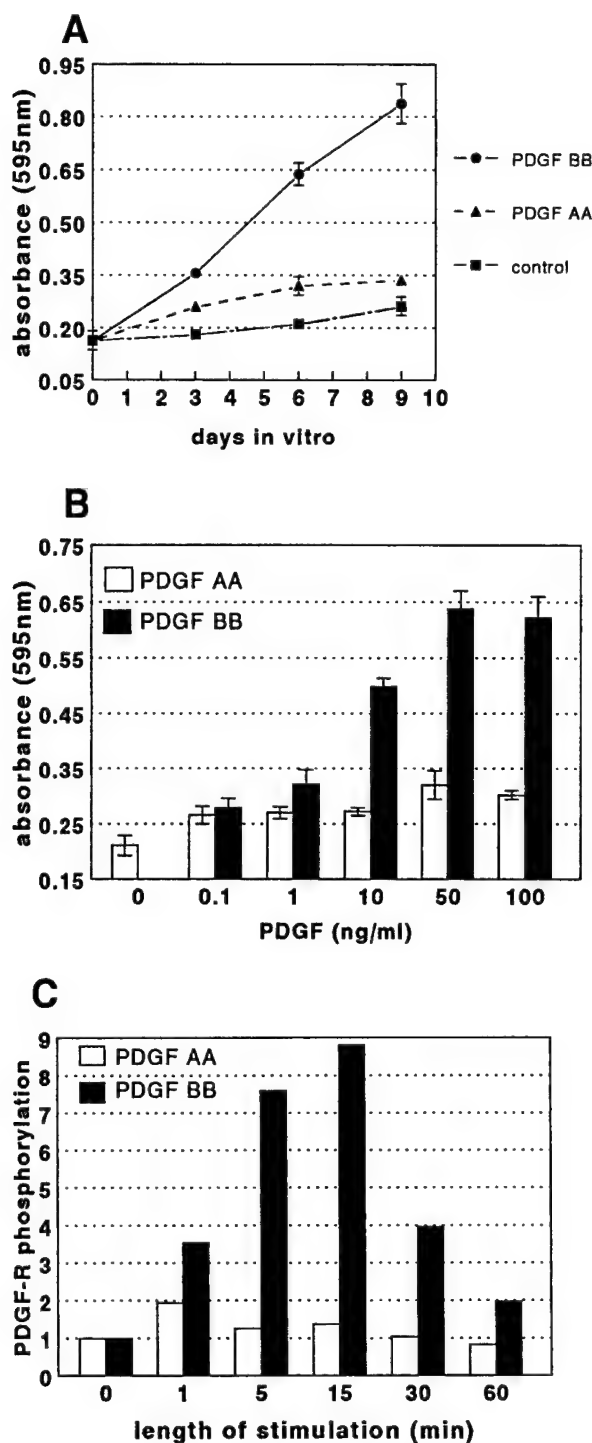
Fig. 6. PDGF AA is a weak mitogen for the T265-2c cell line. **A:** T265-2c cells were cultured in the presence of 50 ng/ml PDGF AA or PDGF BB and cell number evaluated over time as described in Figure 2. **B:** T265-2c cell were cultured for 6 days in the presence of increasing amounts of PDGF AA or PDGF BB and cell number evaluated as described in Figure 2. **C:** Activation of PDGF receptors by PDGF AA and PDGF BB was evaluated in parallel as described in Figure 5. Levels of tyrosyl-phosphorylated PDGF receptor was estimated by densitometric analysis of the band immunostained with the antibody to phosphotyrosine and expressed in arbitrary units. The experiment was repeated twice with similar results.
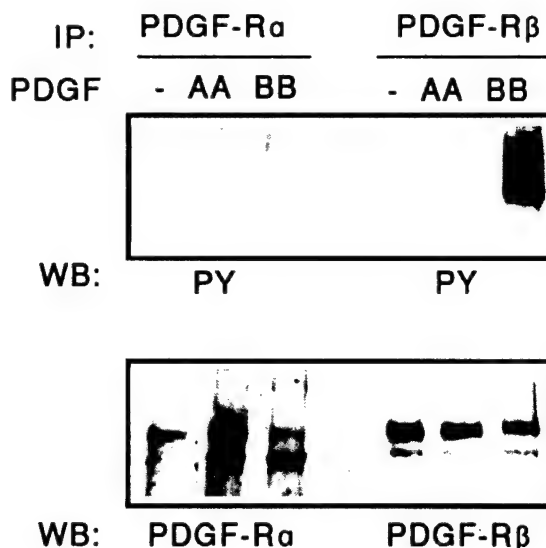
Fig. 7. High levels of activated PDGF receptor β in the T265-2c cell line upon PDGF BB stimulation. T265-2c cells were cultured in control serum-free medium (C) or in the presence of 50 ng/ml of PDGF AA or PDGF BB. After 10 min, cells were lysed and PDGF receptor α or β were immunoprecipitated (IP) using specific antibodies. Immunoprecipitated proteins were analyzed by immunoblotting (WB) using an anti-phosphotyrosine antibody (PY, **upper panel**). The membrane was stripped and the appropriate part of the membrane reprobed with an antibody to PDGF receptor α or β (**lower panel**).

survival and proliferation during development (Morissey et al., 1995; Grinspan et al., 1996) would be eliminated during the transformation process, which generally involves both increased cell growth and decreased cell death.

It is noteworthy that Schwann cells from the *Nf1* knock-out mouse also display decreased responsiveness to another member of the neuregulin family, glial growth factor 2 (Kim et al., 1995). But *Nf1* knock-out mouse Schwann cells show decreased responsiveness to FGF2 as well (Kim et al., 1997), whereas neurofibrosarcoma-derived Schwann cell proliferation is increased by FGF2. Another difference between the two types of cells is the effect of forskolin, which by itself induced hyperplasia of the *Nf1* knockout mouse Schwann cells, but had no effect on proliferation of the neurofibrosarcoma-derived Schwann cells. This difference in behavior between the two *Nf1* knockout cells and the neurofibrosarcoma-derived cells can have several explanations, such as the difference in species, the culture conditions and the status of the transformation process. Indeed, suppression of neurofibromin expression by targeted mutations in the knockout model reproduces early events in the pathologic process leading to the development of NF1. Neurofibrosarcoma-derived cells most probably acquired multiple mutations during their progression toward the malignant phenotype, which may be responsible for the aberrant expression of growth factor receptors or insensitivity to forskolin. Interestingly, these two events may be linked, since it has been shown that forskolin potentiates the mitogenic effect of PDGF BB for Schwann cells, through the induction of PDGF receptor expression (Weinmaster and Lemke, 1990). The fact that, contrary to what

was observed in human and rat Schwann cell cultures, forskolin does not potentiate the mitogenicity of neuregulins, PDGFBB, or FGF2 for neurofibrosarcoma-derived Schwann cells may indicate that cAMP levels in these cells are chronically elevated, which may in turn result in higher expression of growth factor receptors.

PDGF BB is the most potent mitogen for the neurofibrosarcoma-derived Schwann cells. PDGF BB is known to bind both PDGF receptor α and PDGF receptor β (Seifert et al., 1989; Heldin and Westermark, 1990). Immunoprecipitation experiments indicate that, in the neurofibrosarcoma-derived Schwann cells, PDGF BB stimulation results in high levels of activated PDGF receptor β, but low levels of activated PDGF receptor α. PDGF AA, which is known to bind specifically to PDGF receptor α (Seifert et al., 1989), generates similarly low levels of activated PDGF receptor α, and a weak mitogenic response from the neurofibrosarcoma-derived cells. Thus, the increase in PDGF receptor α expression observed in the neurofibrosarcoma-derived Schwann cells may not be large enough to trigger the intracellular signaling pathways involved in proliferation. An estimation of the numbers of PDGF receptors in the T265-2c cell line using NIH-3T3 cells (a commonly used cell type in PDGF receptor studies) as a reference indicate a ratio of PDGF receptor β to PDGF receptor α of 2 to 1. Thus, the lower ratio of PDGF receptor α to PDGF receptor β may explain, in part, the lower level of activation and proliferation induced by PDGF AA. But, the modest effect of PDGF AA on T265-2c cell proliferation may also reflect PDGF receptor α lesser ability to trigger some signaling pathways (Eriksson et al., 1992; Heidaran et al., 1993).

It is now accepted that neurofibrosarcoma initiation and growth are probably the results of multiple sequential mutations involved in the progression toward a malignant phenotype. In addition to mutations in *Nf1,* loss-of-function mutations in tumor suppressor genes such as p53 (Ducatman et al., 1986; Halling et al., 1996) or gain of function mutations in oncogenes such as PDGF receptors, FGF receptors, Kit (Badache et al., 1998), or c-MET (Rao et al., 1997), as well as epigenetic events are likely to participate to the formation of Schwann cell tumors in NF1. Identification of the factors influencing the progression of Schwann cells toward a hyperplastic phenotype could provide new therapeutic strategies for NF1.

ACKNOWLEDGMENTS

LITERATURE CITED

Basu, T.N., Gutmann, D.H., Fletcher, J.A., Glover, T.W., Collins, F.S., and Downward, J. (1992) Aberrant regulation of *ras* proteins in malignant tumour cells from type 1 neurofibromatosis patients. Nature *356:*713–715.

Badache, A., Muja, N., and DeVries, G.H. (1998) Expression of Kit in neurofibromin-deficient human Schwann cells: role in Schwann cell hyperplasia associated with type 1 Neurofibromatosis. Oncogene, *17:*795–800.

Buchberg, A.M., Cleveland, L.S., Jenkins, N.A., and Copeland, N.G. (1990) Sequence homology shared by neurofibromatosis type 1 gene and IRA-1 and IRA-2 negative regulators of the RAS cyclic AMP pathway. Nature *347:*291–294.

Cawthon, R.M., Weiss, R., Xu, G., Viskochil, D., Culver, M., Stevens, J., Robertson, M., Dunn, D., Gesteland, R., O'Connel, P., and White, R. (1990) A major segment of the neurofibromatosis type 1 gene: cDNA sequence, genomic structure, and point mutations. Cell *62:*193–201.

Colman, S.D., Williams, C.A., and Wallace, M.R. (1995) Benign neurofibromas in type I neurofibromatosis (NF1) show somatic deletions of the NF1 gene. Nat. Genet. *11:*90–92.

Davis, J.B., and Stroobant, P.J. (1990) Platelet-derived growth factors and fibroblast growth factors are mitogens for rat Schwann cells. Cell Biol. *110:*1353–1360.

DeClue, J.E., Papageorge, A.G., Fletcher, J.A., Diehl, S.R., Ratner, N., Vass, W.C., and Lowy, D.R. (1992) Abnormal regulation of mammalian p21ras contributes to malignant tumor growth in von Recklinghausen (type 1) neurofibromatosis. Cell *69:*265–273.

Ducatman, B.S., Scheithauer, B.W., Piepgras, D.G., and Reiman, H.M. (1986) Malignant peripheral nerve sheath tumors: A clinicopathologic study of 120 cases. Cancer *57:*2006–2021.

Eccleston, P.A., Funa, K., and Heldin, C.-H. (1993) Expression of platelet-derived factot (PDGF) and PDGF α- and β-receptors in the peripheral nervous system: an analysis of sciatic nerve and dorsal root ganglia. Dev. Biol. *155:*459–470.

Eriksson, A., Siegbahn, A., Westermark, B., Heldin, C.-H., and Claesson-Welsh, L. (1992) PDGF alpha- and beta-receptors activate unique and common signal transduction parthways. EMBO J. *11:*543–550.

Grinspan, J.B., Marchionni, M.A., Reeves, M., Coulaloglou, M., and Scherer, S.S. (1996) Axonal interactions regulate Schwann cell apoptosis in developing peripheral nerve: neuregulin receptors and the role of neuregulins. J. Neurosci. *16:*6107–6118.

Goodearl, A.D.J., Mistry, K., Davis, B.J., Minghetti, L., Otsu, M., Waterfield, M.D., and Stroobant, P. (1993) Purification of multiple forms of glial growth factor. J. Biol. Chem. *268:*18095–18102.

Guha, A., Lau, N., Huvar, I., Gutmann, D., Provias, J., Pawso, and Boss, G. (1996) Ras-GTP levels are elevated in human NF1 peripheral nerve tumors. Oncogene *12:*507–513.

Guy, P.M., Platko, J.V., Cantley, L.C., Cerione, R.A., and Carraway, K.L. (1994) Insect-cell expressed p180erbB3 possesses an impaired tyrosine kinase activity. Proc. Natl. Acad. Sci. U.S.A. *91:*8132–8136.

Halling, K.C., Scheithauer, B.W., Halling, A.C., Nascimento, A.G., Ziesmer, S.C., Roche, P.C., and Wollan, P.C. (1996) p53 expression in neurofibroma and malignant peripheral nerve sheath tumor. Am. J. Clin. Pathol. *106:*282–288.

Hardy, M., Reddy, U.R., and Pleasure, D. (1992) Platelet-derived growth factor and regulation of Schwann cell proliferation in vivo. J. Neurosci. Res. *31:*254–262.

Heidaran, M.A., Beeler, J.F., Yu, J.-C., Ishibashi, T., LaRochelle, W.J., Pierce, J.H., and Aaronson, S.A. (1993) Differences in substrate specificities of α and β platelet-derived growth factor (PDGF) receptors: Correlation with their ability to mediate PDGF transforming functions. J. Biol. Chem. *268:*9287–9295.

Heldin, C.-H., and Westermark, B. (1990) Platelet-derived growth factor: Mechanism of action and possible in vivo function. Cell Regul. *1:*555–566.

Kim, H.A., Ling, B., and Ratner, N. (1997) *Nf1*-deficient mouse Schwann cells are angiogenic and invasive and can be induced to hyperproliferate; reversion of some phenotypes by an inhibitor of farnesyl protein transferase. Mol. Cell. Biol. *17:*862–872.

Kim, H.A., Rosenbaum, T., Marchionni, M.A., Ratner, N., and DeClue, J.E. (1995) Schwann cells from neurofibromin deficient mice exhibit activation of p21ras, inhibition of cell proliferation and morphological changes. Oncogene *11:*325–335.

Lee, J.K., Sobel, R.A., Ciocca, E.A., Kim, T.S., and Martuza, R.L. (1992) Growth of human acoustic neuromas, neurofibromas and schwannomas in the subrenal capsule and sciatic nerve of the nude mouse. J. Neurooncol. *14:*101–112.

Levi, A.D.O., Bunge, R.P., Lofgren, J.A., Meima, L., Heftei, F., Nikolics, K., and Sliwkowski, M.X. (1995) The influence of heregulins on human Schwann cell proliferation. J. Neurosci. *15:*1329–1340.

Li, R.-H., Chen, J., Hammonds, G., Phillips, H., Armanini, M., Wood, P., Bunge, R., Godowski, P.J., Sliwkowski, M.X., and Mather, J.P. (1996) Identification of Gas6 as a growth factor for human Schwann cells. J. Neurosci., *16:*2012–2019.

Marchionni, M.A., Goodearl, A.D.J., Chen, M.S., Bermingham-McDonogh, O., Kirk, C., Hendricks, M., Danehy, F., Misumi, D., Sudhalter, J., Koyabashi, K., Wroblewski, D., Lynch, C., Baldassare, M., Hiles, I., Davis, J.B., Hsuan, J.J., Totty, N.F., Otsu, M., McBurney, R.N., Waterfield, M.D., Stroobant, P., and Gwynne, D. (1993)

Glial growth factors are alternatively spliced erb2 ligands expressed in the nervous system. Nature 362:312–318.

McNiece, I.K., Langley, K.E., and Zsebo, K.M. (1991) Recombinant human stem cell factor (rhSCF) synergizes with GM-CSF, G-SCF, IL-3 and Epo to stimulate human progenitor cells myeloid and erythroid lineages. Exp. Hematol., 19:226–231.

Morissey, T.K., Levi, A.D.O., Nuijens, A., Sliwkowski, M.X., and Bunge, R.P. (1995) Axon-induced mitogenesis of human Schwann cells involves heregulin and p185^{erbB2}. Proc. Natl. Acad. Sci. U.S.A. 92:1431–1435.

Raabe, T.D., Clive, D.R., Neuberger, T.J., Wen, D., and DeVries, G.H. (1996) Cultured neonatal Schwann cells contain and secrete neuregulins. J. Neurosci. Res. 46:263–270.

Rao U.N.M., Sonmez-Alpan, E.N.I., and Michalopoulos, G.K. (1997) Hepatocyte growth factor and c-MET in benign and malignant peripheral nerve sheath tumors. Hum. Pathol. 28:1066–1070.

Riccardi, V.M. (1991) Neurofibromatosis: past, present, future. N. Engl. J. Med. 324:1283–1285.

Rosenbaum, T., Patrie, K.M., and Ratner, N. (1997) Neurofibromatosis type 1: Genetic and cellular mechanisms of peripheral nerve tumor formation. Neuroscientist 3:412–420.

Ryan, J.J., Klein, K.A., Neuberger, T.J., Leftwich, J.A., Westin, E.H., Kauma, S., Fletcher, J.A., DeVries, G.H., and Huff, T.F. (1994) Role for the Stem cell factor/KIT complex in Schwann cell neoplasia associated with neurofibromatosis. J. Neurosci. Res. 37:415–432.

Seifert, R.A., Hart, C.E., Phillips, P.E., Forstrom, J.W., Ross, R., Murray, M.J., and Bowen-Pope, D.P. (1989) Two different subunits associate to create isoform-specific platelet-derived growth factor receptors. J. Biol. Chem. 264:8771–8778.

Tzahar, E., Pinkas-Kramarski, R., Moyer, J.D., Klapper, L.N., Alroy, I., Levkowitz, G., Shelly, M., Henis, S., Eisenstein, M., Ratzkin, G.C., and Yarden, Y. (1997) Bivalence of EGF-like ligands drives the erbB signalling network. EMBO J. 16:4938–4950.

Viskoshil, D., Buchberg, A.M., Xu, G., Cawthon, R.M., Stevens, J., Wolf, R.K., Culver, M., Carey, J.C., Copeland, N.G., Jenkins, N.A., White, P., and O'Connell, P. (1990) Deletions and a translocation interrupt a cloned gene at the neurofibromatosis type 1 locus. Cell 62:187–192.

Wallace, M.R., Marchuk, D.A., Andersen, L.B., Letcher, R., Odeh, H.M., Saulino, A.M., Fountain, J.W., Brereton, A., Nicholson, J., Mitchell, A.L., Brownstein, B.H., and Collins, F.S. (1990) Type 1 neurofibromatosis gene: Identification of a large transcript disrupted in three NF1 patients. Science 249:181–186.

Weinmaster, G., and Lemke, G. (1990) Cell-specific cyclic AMP-mediated induction of the PDGF receptor. EMBO J. 9:915–920.

Xu, G., O'Connell, P., Viskochil, D., Cawthon, Robertson, M., Culver, M., Dunn, D., Stevens, J., Gesteland, R., White, P., and Weiss, R. (1990) The neurofibromatosis type 1 gene encodes a protein related to GAP. Cell 62:599–608.

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theme letter: B
1st topic title: NEUROGLIA & MYELIN topic number: 26

2nd theme title: DEVELOPMENT & REGENERATION theme letter: A
2nd topic title: PROLIFERATION
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Key Words: (see instructions p. 4)

Prostaglandins elevate intracellular cAMP levels in Schwann cells.
N Muja[1]* and GH DeVries[2,3]
[1]Program in Neural Science and [2]Department of Cell Biology, Neurobiology, and Anatomy, Loyola University of Chicago, Maywood, IL 60153
[3]Research Service, Edward J Hines VA Hospital, Hines, IL 60141

Following peripheral nerve injury, hematogenous macrophages are rapidly recruited to the injury site where they phagocytize myelin and axonal debris. Activated macrophages are also known to release prostaglandins which are locally acting hormones derived from arachidonic acid by the actions of cyclooxygenases. Prostaglandins activate receptors coupled to G proteins producing an elevation in intracellular cAMP and/or calcium. Given that macrophages produce prostaglandins specifically at the site of injury, it seems likely that Schwann cells located within or near the injury site will be physiologically responsive to prostaglandin stimulation. We hypothesize that Schwann cells express prostaglandin receptors coupled to adenylyl cyclase. Using RT-PCR, we demonstrate that primary rat Schwann cells express RNA transcripts encoding the EP2, EP4, and IP prostaglandin receptors. Using a competitive ELISA specific for cAMP, we discovered that prostaglandin receptor stimulation resulted in a rapid, dose dependent elevation in intracellular cAMP levels. To confirm our ELISA findings, we performed an immunocytochemical analysis of intracellular cAMP levels using an antibody and staining procedure developed by Weimelt et al. (J Biol Chem 272(50): 31489-95). We demonstrate that, compared to forskolin treated Schwann cells, a small subset of the Schwann cell population was strongly immunoreactive for cAMP following prostaglandin treatment and that this subset of cells displayed a morphology suggestive of a differentiated phenotype. Current studies are underway to further characterize the signal transduction events that are initiated following prostaglandin stimulation and their role in Schwann cell detachment and proliferation during Wallerian degeneration. (Supported by Arthur J Schmidt Fellowship awarded to NM and a Hines VA Medical Research Service grant awarded to GHDV).

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1st topic title: Neuro-oncology topic number: 138

2nd theme title: Cell Biology theme letter: B
2nd topic title: Neuroglia and Myelin topic number: 26

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Key Words: (see instructions p. 4)

Neurofibrosarcoma-Derived Schwann Cells Have Decreased RAS-GAP and Elevated cAMP levels. [1]Ian Dang, [2]Simeng Suy, [1]G.H. DeVries[*] [1]Hines VA Hospital, Hines, IL 60141/ Dept of Cell Biology, Neurobiology, and Anatomy. Loyola University. Maywood, IL 60153 and [2]Dept of Radiation Medicine, Georgetown University, Washington DC 20007.
Neurofibromatosis Type 1 (NF1) is a human genetic disorder affecting approximately 1 in 3000 individuals that manifests with various phenotypic features including café-au lait spots, and neurofibromas, which are benign peripheral nerve sheath tumors primarily made up of Schwann cells. These benign tumors can become malignant neurofibrosarcomas. Hyperplasia in NF1 is primarily due to the mutated GAP domain of the neurofibromin protein resulting in high levels of activated RAS (RAS_{GTP}). The extent to which alterations in non-NF GAP contribute to this elevated RAS_{GTP} is not known. We report that two neurofibrosarcoma-derived cell lines (T265 and ST88) have a three to four fold lower expression of RAS-GAP relative to a non-malignant Schwannoma cell line (STS26T). We have reported other abnormalities in these neurofibrosarcoma cell lines include high expression of PDGF receptors (J. Cell. Phys. 177:334, 1998). We investigated levels of intracellular cAMP in these cell lines, since growth factor receptor expression in Schwann cells is regulated by intracellular cAMP. Relative to the Schwannoma cell line or neonatal human Schwann cells, intracellular levels of cAMP in the neurofibrosarcoma-derived Schwann cells are 6 to 10 fold elevated. These low levels of GAP and elevated intracellular cAMP levels support the following paradigm: mutations in the GAP domain of neurofibromin as well as lowered expression of other GAP proteins lead to elevated levels of activated RAS. In turn, the high levels of RAS_{GTP} lead to increased concentrations of intracellular cAMP which then increase PDGF receptor expression. In concert, these changes in intracellular transduction pathways lead to the Schwann cell hyperplasia characteristic of NF1 (Supported by US Army Medical Research and Material Command; Contract grant number: DAMD17-98-8607).

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Identification and Functional Characterization of Thromboxane A2 Receptors in Schwann Cells.

SC Blackman[1], N Muja[2], GC LeBreton[1], GH DeVries[3,4]
[1]Department of Pharmacology, University of Illinois at Chicago, Chicago, IL, 60612
[2]Neuroscience Graduate Program, Loyola University of Chicago, Maywood, IL, 60153
[3]Research Service, Hines VA Hospital, Hines, IL, 60141
[4]Department of CBNA, Loyola University of Chicago, Maywood, IL, 60153

Previous work has demonstrated the presence of thromboxane A2 (TxA2) receptors in neonatal rat oligodendrocytes and human oligodendroglioma cells. In these experiments, the presence and function of TxA2 receptors in neonatal rat Schwann cells (rSC) and a human neurofibrosarcoma-derived Schwann cell line (T265-2c) was investigated. Immunocytochemical analysis using polyclonal antibodies raised against purified TxA2 receptor demonstrated that rSC express TxA2 receptors. Similarly, immunoblot analyses of electrophoretically resolved proteins from rSC revealed that rSC express a 55 kDa protein which was immunoreactive with several anti-TxA2 receptor antibodies, commensurate with previous results obtained using oligodendrocytes and human blood platelets. To test the functionality of TxA2 receptors in rSC and the T265-2c cell line we monitored intracellular calcium responses using Fura-2 fluorescence. In addition, we monitored changes in intracellular cAMP levels using a sensitive enzyme-linked immunoabsorbent assay. Treatment with U46619, a stable thromboxane A2 mimetic, did not stimulate intracellular calcium mobilization in rSC whereas the T265-2c cell line demonstrated a calcium response. In contrast, treatment with 5uM U46619 for 5 minutes resulted in a significant increase in intracellular cAMP levels in both cell types. In sum, these results demonstrate that rSC express a thromboxane A2 receptor protein of similar electrophoretic mobility to that found in platelets and oligodendrocytes. Furthermore, receptor stimulation using U46619 produced an elevation of intracellular cAMP in both cell types and an increase in intracellular calcium levels in only the T265-2c cell line. Investigations are in progress to determine the functional role of TxA2 receptors in both normal Schwann cell physiology and Schwann cell hyperplasia associated with Neurofibromatosis type 1. (Supported by Department of the Army #DAM17-98-01-860 awarded to GHD.)

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protein/hour, increased to 5.4 $\pm$ 0.87 at 6 hours and began decreasing at each time point reaching a low of 2.1 $\pm$ 0.04 nmoles/mg protein/hour at 7 days which persisted for 35 days. When cells treated for 7 days with dBcAMP were switched to dBcAMP-free medium, uptake rate increased to 2.9 $\pm$ 0.09 nmoles/mg protein/hour within 24 hours. It is concluded that dBcAMP downregulations *myo*-inositol uptake and that this effect is partially reversible. It appears that in addition to osmotic stress and *myo*-inositol deprivation, that factors associated with differentiation also influence the number of *myo*-inositol transporters in cultured astrocytes.

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C

RETROVIRAL TRANSFORMATION OF HUMAN FETAL SCHWANN CELLS WITH AN INDUCIBLE *MYC* CONSTRUCT CONFERS HORMONE-DEPENDENT PROLIFERATION.

T. J. LOPEZ AND G. H. DE VRIES. Department of Cell Biology, Neurobiology, and Anatomy, Loyola University Medical Center, 2160 South 1ˢᵗ Avenue, Maywood, IL, 60153, and Hines VA Hospital, Research (151), Hines, IL, 60141.

Human fetal Schwann cells (SC) were isolated from gestational week 17-19 sciatic nerves, purified, and maintained under serum-free conditions in the presence of 10 ng/ml NDFβ. The amphotropic PA317 packaging cell line was transfected with a pMV7-derived plasmid containing the chimaeric protein mycER, consisting of the hormone-binding portion of the human estrogen receptor and *myc*. Transfected PA317 cells producing retrovirus were selected in ampicillin-resistant DMEM/10% serum. Supernatant containing retrovirus was collected and used for transformation of SC at 1:1000 to 1:10,000 dilutions. SC were incubated with retrovirus four hours, grown for two days in DMEM / 5% serum, and then maintained in neomycin selection medium (DMEM, 5% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mg/ml neomycin sulfate). We find that transformed SC express *mycER*, and proliferate in response to 2 µM estrogen in defined (N2) culture medium. The hormone-dependent proliferation of these SC can be ameliorated by culture in N2 medium that is free of serum, estrogen, and phenol red; under these conditions transformed SC survive, become quiescent, and express most protein markers characteristic of normal human fetal SC. Transformed SC also proliferate when grown in the presence of fetal bovine serum, owing to the high concentration of nascent β-estrogen in fetal serum. Interestingly, we could not detect the erb B receptors on transformed SC immediately following estrogen removal, and SC newly expanded with estrogen did not respond to the neuregulin NDFβ. In summary, this inducible expression vector system provides a highly efficient method for generating stable transformed human SC. Transformed SC expanded by hormonal induction of *mycER* become phenotypically similar to normal SC following removal of estrogen, providing an excellent source of human SC for study. (Supported in part by grants from the Massachusetts Bay Area Neurofibromatosis, Inc., the Illinois NF Society, and the Department of the Army #DAM17-98-01-860 to GHDV and from NIH grant FS NS10164 to TJL.)

D

NON-LETHAL DETECTIO
IN THE CNS OF FOUNDE:

T. Wakabayashi*, A. Messin;
*Stroke Branch, NINDS, NIF
†Department of Pathobiologic
Medicine and the Waisman C
Madison, 1500 Highland Ave
ᶜDepartment of Neurobiology
University of Alabama at Biⁿ
0021

Determining whether tran;
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generate offspring from the
We describe a method for as;
CNS-targeted transgenes that
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have been used to direct tran
show that this method read
specific *GFAP*-driven *lacZ* tr.
lacZ transgene.

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1st topic title: Neuroglia and myelin
topic number: 27

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EXPRESSION OF HIGH LEVELS OF FUNCTIONAL PLATELET-DERIVED GROWTH FACTOR RECEPTORS IN NEUROFIBROSARCOMA-DERIVED SCHWANN CELLS <u>Ali Badache and George H. DeVries</u>*. Department of Cell Biology, Neurobiology and Anatomy, Loyola University Medical Center Chicago and Hines VA Hospital, Research 151, Hines, IL 60141.

Neurofibromas, hallmark features of Neurofibromatosis type 1 (NF1), are benign tumors of the peripheral nerve composed mainly of Schwann cells. In about 5 % of NF1 patients, malignant tumors called neurofibrosarcomas arise from the neurofibromas, through a yet unknown mechanism. We show here that levels of expression of both platelet-derived growth factor (PDGF) receptors α and β are strongly increased in two neurofibrosarcoma-derived cell lines compared to a Schwann cell line derived from a schwannoma (from a non-NF1 patient) and to immortalized rat Schwann cells. PDGF BB strongly stimulates the proliferation of the neurofibrosarcoma-derived Schwann cells cultured in serum-free medium, in a dose-dependent manner. PDGF BB is not mitogenic for the non-NF1 Schwann cell lines. Mitogenicity of most Schwann cell mitogens is greatly improved in the presence of elevated intracellular cAMP. Interestingly, forskolin, which is known to increase intracellular cAMP, does not potentiate the mitogenic effect of PDGF BB for neurofibrosarcoma-derived Schwann cells. Even though expression levels of PDGF receptor α are elevated in the neurofibrosarcoma-derived Schwann cell lines, PDGF AA is only a weak mitogen for these cells by comparison to PDGF BB. Levels of tyrosyl-phosphorylated PDGF receptor β is strongly increased upon stimulation by PDGF BB. In comparison, only modest levels of tyrosyl-phosphorylated PDGF receptor α are observed, upon stimulation by PDGF AA or PDGF BB. Thus, aberrant expression and activation of growth factor receptors by Schwann cells, such as the PDGF receptors, could play a role in the process leading to Schwann cell hyperplasia in NF1 (supported by Mass. Bay Area Neurofibromatosis, Inc. and the Illinois Neurofibromatosis, Inc.).

Key Words: (see instructions p. 4)

1. Schwann cells
2. Growth Factors
3. Neurofibromatosis
4. Tyrosine kinase

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Thomas J. Lopez, Ph. D.

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RETROVIRAL TRANSFORMATION OF PRIMARY HUMAN FETAL SCHWANN CELLS. T.J. Lopez* and G.H. De Vries. Department of Cell Biology, Neurobiology, and Anatomy, Loyola University Medical Center, 2160 South First Avenue, Maywood, IL, 60153, USA, and Research Service (151), Edward Hines Jr. VA Hospital, 5th Avenue and Roosevelt Road, Hines, IL, 60141, USA.

Human fetal Schwann cells (HFSC) were isolated from peripheral (sciatic) nerve of gestational week 17-19 concepti. HFSC were plated on laminin-coated plastic dishes, and enriched cultures of HFSC were obtained by maintenance under serum-free conditions in the presence of the neuregulin NDFβ. A retroviral packaging cell line PA317 (ATCC) was transformed using the Superfect Transfection Reagent Kit (Qiagen) according to manufacturers' instructions with a pMV7-derived plasmid containing the chimaeric protein mycER, consisting of the hormone-binding portion of the human estrogen receptor and *myc*. Retrovirus particles were collected in the supernatant of transfected PA317 cells, and purified HFSC were transformed by two hour exposure to *mycER* retrovirus. Transformed HFSC cultures were grown in selection medium (DMEM, 10% fetal bovine serum, 5 U/ml penicillin, 5 µg/ml streptomycin, 2 mg/ml neomycin) to select for transformed HFSC. The hormone-dependent proliferation of these HFSC can be ameliorated by culture in phenol red-free DMEM supplemented with steroid-depleted fetal bovine serum. Under these conditions, transformed HFSC become quiescent and express a phenotype characteristic of untransformed HFSC. Transformation of cells with this inducible retroviral expression vector provides a stable and a renewable source of HFSC that can be expanded at will upon hormonal induction of the retroviral *mycER* chimaeric protein with estrogen or estrogen analogs. (Supported in part by grants from the Massachusetts Bay Area Neurofibromatosis, Inc. and the Illinois NF Society to GHDV and from NIH grant FS NS10164 to TJL.)

Key Words: (see instructions p. 4)

1. Schwann cell ... Culture
2. Glia

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1st topic title: Glia and other non-neuronal cells   topic number: 18

2nd theme title: Cell Biology   theme letter: B
2nd topic title: Neuroglia and myelin   topic number: 27

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DEVELOPMENTAL REGULATION OF C-KIT EXPRESSION IN SCHWANN CELLS. I. Dang, A. Badache, T. J. Lopez*, and G. H. DeVries. Dept of Cell Biology, Neurobiology, and Anatomy. Loyola University. Maywood, IL 60153 / Hines VA Hospital, Hines, IL 60141.

We have reported that Schwann cells derived from neurofibromas, benign peripheral nerve sheath tumors, abnormally express high level of the receptor tyrosine kinase c-kit, which may contribute to Schwann cell hyperproliferation. This observation raises the possibility that c-kit has important roles during the normal development of Schwann cells. We report that c-kit is expressed in rat sciatic nerves and that c-kit expression is developmentally regulated: c-kit expression is high in sciatic nerve of rat pups between postnatal day 0 and 10, which corresponds to a phase of Schwann cell proliferation in the sciatic nerve. The expression of c-kit decreases dramatically after day 10 and remains low in adult sciatic nerves. In contrast, the expression of a major myelin protein (P0) is low in sciatic nerves of postnatal day 0 rat pups and increases to reach a maximum at postnatal day 16, and persists elevated in adult rats. In order to determine the cells responsible for the developmental regulation of c-kit expression in rat sciatic nerves, we analyzed Schwann cell and fibroblast cultures for c-kit expression by RT/PCR and western blots: c-kit mRNA and protein were detected in Schwann cells but not in fibroblasts. In conditions that favor proliferation (20μM 8-Br cAMP), Schwann cells display high levels of c-kit expression; however, under conditions which favor differentiation (1mM 8-Br cAMP), Schwann cells display low levels of c-kit expression. Therefore, high c-kit expression is associated with proliferation, whereas the expression of c-kit is minimal when Schwann cells differentiate. These results suggest that c-kit may play a role in Schwann cell proliferation during development and in Schwann cell hyperplasia associated with pathological conditions, such as neurofibromatosis (supported by Neurofibromatosis, Inc, Mass Bay Area).

Key Words: (see instructions p. 4)

1. Proliferation
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ROLE OF c-KIT IN THE ETIOLOGY OF TYPE 1 NEUROFIBROMATOSIS

Badache A. and DeVries G.H., Loyola University Medical Center of Chicago/ Hines VA Hospital, Hines IL 60141

Neurofibromatosis type 1 (NF1) is a genetic disease characterized by the development of neurofibromas. Neurofibromas are tumors of the peripheral nerves, composed mostly of Schwann-like cells. Even though mutations in a gene coding for a tumor suppressor protein (neurofibromin) have been detected in patients with NF1, the mechanism leading to hyperplasia of Schwann cells in neurofibromas remains largely unknown. We have described the expression of the proto-oncogene Kit, a tyrosine kinase receptor, in Schwann cell lines derived from neurofibromas, as opposed to normal human and rat Schwann cells. Since Schwann cells can express the ligand for Kit (Stem Cell Factor), we hypothesized that an autocrine loop involving Kit and its ligand could be part of the mechanism leading to NF1-Schwann cell proliferation. Kit expression is increased only in cell lines with decreased neurofibromin expression : Kit was not overexpressed in a Schwannoma-derived cell line, nor in a neurofibroma-derived cell line which maintained characteristics of non-transformed cells, including normal levels of neurofibromin. Since Kit was present in the cell lines in the tyrosyl-phosphorylated form, it could potentially transduce a proliferative signal to the cells. Tyrosine kinase inhibitors induced a dose-dependent decrease in NF1-cell proliferation, that correlated with a general decrease in protein tyrosyl-phosphorylation. The most efficient inhibitor of NF1-Schwann cell proliferation was Tyrphostin A9, which has been reported to be specific for the subclass III receptors, which include Kit. In order to directly demonstrate that Kit activity is involved in neurofibroma-derived Schwann cell proliferation, the ST88-14 cell line was transfected with a truncated dominant negative (DN) form of Kit. The proliferation rates of the DN-transfected cells were generally decreased compared to control cells; the extent of the inhibition is expected to correlate with the level of DN-Kit transcript expressed. Anchorage-independent growth of the DN-transfected cell lines was impaired as well. These data are consistent with an important role for Kit in the growth of neurofibroma-derived cell lines. The presence of high levels of Kit in neurofibromas, as demonstrated by immunohistochemistry, argues for a similar role for Kit *in vivo*. We are currently investigating whether the increased expression of Kit is the direct result of neurofibromin deficiency or an independent phenomenon required for NF1-Schwann cell hyperplasia. (Supported by Neurofibromatosis, Inc., Mass Bay Area).

Neurofibromatosis

Schwann cells

c-Kit

Oncogene

Tyrosine kinase

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PURIFICATION AND FUNCTIONAL ANALYSIS OF PRIMARY FETAL HUMAN SCHWANN CELLS. T. J. Lopez, M. F. Dauzvardis*, and G. H. De Vries. Dept. of Cell Biology, Neurobiology, and Anatomy, Loyola University Medical Center, Maywood, IL, 60153, USA, and Research Service, Hines VA Hospital, Hines, IL, 60141, USA.

We have developed methodologies useful for isolating large numbers of fetal human Schwann cells (FHSC) and obtaining highly pure cultures of FHSC *in vitro*. The FHSC originate from fetal peripheral nerve samples 18-20 weeks *in utero*. Our methods give a high cell yield of viable cells ($2.69\{\pm0.30\} \times 10^6$ living cells per 100 mg of tissue), and the number of contaminating fibroblasts is less than when SC are isolated and recovered from adult human nervous tissue. Fibroblasts were eliminated from FHSC cultures by cycling with AraC, and immunocytochemical staining was used to characterize primary FHSC. We find: *(a)* FHSC are bipolar, spindle-shaped, and align in fascicles similar to adult human SC; *(b)* fibroblasts can be eliminated from these primary FHSC cultures more readily than from adult human primary SC cultures, where they persist without addition of mitogens; *(c)* primary FHSC are immunoreactive with antibodies to S100, MBP, and P_0, and also are O1 and O4 positive; *(d)* these FHSC express the early glial cell markers GFAP and cKIT, as well as the low affinity nerve growth factor receptor, LNGFR, and do not express laminin; and *(e)* FHSC cultured *in vitro* can myelinate fetal human dorsal root ganglion neurites *in vitro*. These results indicate that FHSC can be obtained and cultured for months *in vitro*, morphologically resemble adult human SC but lack the mature SC immunocytochemical phenotype, however these FHSC are myelin-competent. This method of FHSC isolation and the *in vitro* system for assessing functional capacities of these primary FHSC provide valuable tools for understanding the development of normal human SC. (Supported in part by NIH NS10821.)

Key Words: (see instructions p. 4)

1. Schwann Cell (human)
2. Differentiation
3. Myelin
4. Tissue Culture

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